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THE EFFECT OF HARDNESS, ALKALINITY AND pH ON

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THE EFFECT OF MUCOUS SECRETION ON

CUPRIC ION ACTIVITY AND pH, AND

A POSSIBLE INTERNAL MODE OF

COPPER TOXICITY

by

Ⓒ THERON G. MILLER

A THESIS

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies and Research, for
addeptance, a thesis entitled " The Effects of Hardness, Alkalinity
and pH on the Toxicity of Copper to Rainbow Trout, The Effect of
Mucous Secretion on Cupric Ion Activity and pH, and a Possible
Internal Mode of Copper Toxicity" submitted by Theron G. Miller
in partial fulfilment of the requirments for the degree of
Master of Science.

ABSTRACT

Fifteen day toxicity tests were conducted in an artificial fresh water medium to determine the incipient (threshold) LC50 (ILC50) for copper to 8-15 g _Salmo_ _gairdneri_ in various combinations of water hardness (12 to 100 mg/1) and alkalinity (10 to 50 mg/1), in several concentrations of hydrogen ion (pH 4.35 to 7.3) and after exposure to sublethal concentrations of copper and acid. Total and soluble copper were measured by atomic absorption spectrophotometry. The dry weight of mucus secreted and sloughed off in 8 hours into a 2 liter holding tank by trout exposed to copper and acid was measured. In addition, the influence of mucus on cupric ion activity was measured by titrating a standard solution of copper with mucus solution against a copper standard. These titrations were conducted at several pHs (3.0 to 7.3). Cupric ion activity was measured with a cupric ion specific electrode.

Results of these experiments showed that calcium is much more important than alkalinity in reducing copper toxicity. At low water hardness (12 mg/1) the ILC50 of copper was not affected by a five fold change in medium alkalinity (10 to 50 mg/1). At low alkalinity (10 mg/1) increasing the hardness from 12 to 98 mg/1 caused a three-fold increase in the ILC50. However in hard water (100 mg/1) increasing the alkalinity from 10 to 50 mg/1 caused the ILC50 to increase by 1.8 times. The ILC50 for copper increased with increasing water hardness regardless of alkalinity. At pH 5.4 there was synergism between acid pH and copper but at pHs of less than 5.4 the toxicity of various combinations of copper and hydrogen ion was antagonistic.

After one week of exposure to a sublethal concentration of copper (25-30 ug/l) the ILC50 (96 ug/l) was nearly twice that of fish not previously exposed to copper. However, trout held in sublethal acid conditions (pH 4.9-5.2) for one week showed no acclimation to acid pH.

Excessive mucus secretion was not found to occur as a result of exposure to 80 ug/l copper for 8 hours. However mucus secretion increased by 30% compared to control values in response to 8 hours of exposure to pH 4.0. Titrations with a mucus solution revealed that mucus efficiently chelated copper even down to pH 3.5. In addition, each of 3 80-100 g fish raised the pH of 2 liters of water from 4.0 to 6.5 in 8 hours. The excessive mucus secretion which occurred at low pH's coupled with the ability of mucus to chelate copper and buffer acid could be responsible for the antagonism or protecting effect that acid showed when combined with copper.

An in-vitro preparation of the frog (Rana pipiens) rectus abdominus muscle was used to test the effect of copper on muscle activity. It was attached to a muscle transducer-electrophysiograph apparatus. After exposure for 20 minutes to 10^{-6}M (64 ug/l) copper (10^{-5}M)) acetylcholine induced rapid convulsive-like contractions. As this activity was observed in fish very near death in the toxicity tests these result suggest an internal mode of toxicity at the sensitive incipient LC50 level.

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INTRODUCTION

The water draining from regions where copper ore is exposed to weathering and leaching often contains elevated levels of copper and has a low pH. The low pH results from oxidation and hydration of sulfides associated with copper bearing rock (Spaulding and Ogden, 1968). Although there is an abundance of literature demonstrating the extreme toxicity of copper (Lloyd, 1961; Lloyd and Herbert, 1962; Sprague, 1964; Sprague and Ramsey, 1965; Van Collie and Jones, 1974; McKim and Benoit, 1971; McKim and Benoit, 1974; Brungs et al., 1975; Lorz and McPherson, 1976; Lett et al., 1976) and low pH (EIFAC, 1969; Beamish, 1974; Lloyd and Jordan, 1964; Daye and Garside, 1975; Menendez, 1976; Dunson and Martin, 1973; Craig and Baksi, 1977; Trojnar, 1977) to fish, the interactions between these two toxicants has not been thoroughly investigated.

Copper and low pH appear to be similar in their mechanisms of toxicity. Lorz and McPherson (1976) reported that exposure of freshwater coho salmon (Oncorhynchus kisutch) smolts to copper significantly reduced gill sodium-potassium ATPase activity. This reduction was believed to cause a loss of osmoregulatory ability as indicated by death when the smolts were transferred from freshwater to sea water. Copper strongly inhibits the influx of chloride in frog skin (Ussing, 1949; Zadunaisky et al., 1963) and Parisi and Paccinni (1972) found that copper had an inhibitory effect on the hydrosmotic response of the toad

bladder to oxytocin. The golden shiner (Notomigonus crysoleucas) and striped bass (Roccus saxalitis) have been found to suffer from osmotic stress, showing a reduction in blood osmotic pressure as a result of copper exposure (Lewis and Lewis, 1971). Acid pH has been shown to cause a decrease in plasma osmolality due to decreased sodium influx and increased sodium efflux in brook trout (Salvelinus fontinalis) (Packer and Dunson, 1970). Both copper and acid pH have been reported to cause excessive mucous secretion and precipitation on the gills with death being attributed to suffocation (Ellis, 1937; Westfall, 1945; Carpenter, 1927; Plonka and Neff, 1969). Finally, similar injuries to gill tissue have been reported by Daye and Garside (1976) and Plonka and Neff (1969) for acid and Baker (1969) for copper.

The speciation of copper at various concentrations of alkalinity and values of pH has been described by Stiff (1971a) and Sylva (1976). They report that inorganic alkaline sub-stances bind Cu^{++} in neutral and high pH ranges and decreasing the pH liberates increasing percentages of cupric ion from these complexes. Therefore, it was postulated that the observed differences in copper toxicity in test waters of different hardness is due to the degree of Cu^{++} binding with inorganic anions which vary directly with hardness. This is theoretically supported by the work of Pagenkopf et al. (1974) who conducted simultaneous equilibrium computations for various species of copper using water chemical data from various published tox-icity tests. Their calculations indicated that copper

toxicity is directly linked with the concentration of cupric ion. This was further supported by the experiments of Shaw and Brown (1974) where dilution of a hard, alkaline water to obtain various hardness, alkalinity, and pH values linked copper toxicity to cupric ion and possible CuCO3 concentrations. Similarly, Andrew et al. (1977) found that addition of small amounts of carbonates and inorganic phosphates increased the median survival time of Daphnia magna in lethal concentrations of copper.

Because of the similar histological and physiological effects of copper and acid and the evidence that reducing the pH liberates the toxic cupric ion from inorganic complexes, the toxicity of combinations of acid and copper would be expected to be synergistic or potentiated. Therefore, because of the common occurrence of these two pollutants together the first objective of this study was to experimentally evaluate their toxic interaction.

In addition, although there is convincing evidence that alkalinity is the major factor in modifying copper toxicity, there is some evidence (Lloyd, 1965) that calcium has a protecting effect on the fish. Furthermore, this protection may last for a few days after transfer to soft water (Lloyd, 1965). Thus the second objective of this study was to determine the importance of calcium in modifying copper toxicity.

Several authors have demonstrated that fish acclimatize to sublethal concentrations of heavy metals. This was first re-

ported by Paul (1952) and Grande (1967) where they found that water containing heavy metals supported endemic populations of trout while transplanted rainbow trout died. Since that time, O'Hara (1971) found that bluegill sunfish (Lepomis macrochirus) showed an acclimatory response in oxygen consumption with exposure to copper. McKim et al. (1970) and Christensen et al. (1972) showed that exposure to sublethal concentrations of copper induced transient effects on several blood parameters, including hematocrit, glucose, protein and osmolarity, in brook trout and brown bullheads (Ictalurus nebulosus) Donaldson and Dye (1975) found a transient effect on plasma corticosteroid concentrations at all copper concentrations which did not cause death within 24 hours. Finally, Lett et al. (1976) demonstrated an acclimatory response in appetite and growth rate for rainbow trout exposed to copper.

In view of these observations it is possible that the observed differences in copper toxicity in water of different chemical characteristics may also be a result of exposure to various background levels of heavy metals. It seems possible that previous exposure to even very low concentrations of heavy metals may increase the fishes' tolerance to potentially lethal concentrations. Therefore, a third objective of the present study was to assess the ability of rainbow trout to acclimate to copper.

Excessive mucous secretion in response to soluble heavy metals and extreme acid pH values have been reported by several authors (Carpenter, 1927; Ellis, 1937; Jones, 1938; 1939; Westfall, 1945; Plonka and Neff, 1969). These authors reported

that the mucus coagulates and precipates with the metal or hydrogen ion on the gill surface and causes subsequent death by suffocation. More recently, several authors (Lloyd, 1965; Skidmore, 1970; Skidmore and Tovell, 1972; Jones, 1964) have observed histological damage to gill tissue involving separation of the epithelium from the basement membrane with copper and zinc poisoning. This separation is postulated to cause suffocation by increasing the diffusion distance for respiratory gases between water and blood. These observations were made on fish exposed to concentrations of toxicants ranging from approximately 2 to 30 times the ILC50. Recently, Sellers et al. (1975) exposed rainbow trout to copper and zinc concentrations ranging downward from the 48 hour LC50 and found that there was no excessive accumulation of mucus with copper exposure but mucus accumulation was readily apparent in zinc exposed fish. In addition, these authors monitored blood PO_2 and pH in these fish and found that copper exposure caused no change in PO_2 or pH after 24 hours and only a moderate reduction in PO_2 after 86 hours. However, zinc exposure caused a significant reduction in blood PO_2 and pH. Thus although similar histological deformities occur among metals, the physiological effects of this damage may not be the same.

The histological damage observed by Plonka and Neff (1969) with brook trout exposed to extremely low pH values was similar to that seen following exposure and heavy metals. In addition, they found bits of cellular debris such as nuclei and membrane fragments, which apparently resulted from the destruc-

tion of mucous cells embedded in a mucous matrix. They suggest-
ed that the coagulum is formed by cellular debris adhering to
the respiratory epithelial cells.

Labat et al. (1974) using the light microscope observed
that exposure to near lethal concentrations of copper for 24 or
48 hours caused atrophy of mucous cells. Upon return to clean
water, mucous cells again appeared within 24 hours at a similar
density as in control fish. They concluded that the mucous
cells had not been destroyed but they had ejected all of their
contents and any further manufactured mucus was immediately
ejected. Very few mucous particles were found to adhere to the
surface of the lamellae. Thus, there is evidence that excessive
mucous secretion is sloughed off freely and only at extremely
high concentrations of heavy metals or H+, where extensive
tissue damage is done, does a coagulation film occur. This
appears to be especially true for copper exposure. Thus, a
fourth objective of the present study was to investigate mucous
secretion rates in response to copper and acid exposure.

In most toxicity tests, the rate at which test solution
flows through the test tank (rate of exposure of the toxicant to
the fish) is much less than that in a stream or lake system.
With this unavoidable and significant difference in flow rates,
toxicity testing chambers accumulate a certain amount of dis-
solved, suspended, and sedimented organic matter including
mucous secretions and excretory products. These organic sub-
stances may play a significant part in dictating the speciation
of copper. Because mucus is primarily composed of protein

(Pickering, 1976; Wessler and Werner, 1957), it may have a significant effect on the speciation of copper. Peisach et al. (1967) discusses in great detail the different binding sites and subsequent complexes various proteins form with copper. Stiff (1971b) demonstrated that the complexing of copper by single amino acids and small peptides at 10^{-4} M (1.4 mg/l as N) forms the most abundant species of copper in natural water except in extremely alkaline waters where $CuCO_3$ is the most abundant species. Therefore, because of the potential importance of proteins, peptides, and amino acids in determining the speciation of copper and subsequently rendering the copper non-toxic, the observed increase in mucous secretion and resulting accumulation of mucus in the testing chambers may significantly affect the speciation and toxicity of copper.

In addition, because of the apparent ability of protein to bind cations and their common occurrence as anions in biological systems, it is also possible that mucus may act as a buffer against acidic conditions.

Investigations into the possible internal mode of copper toxicity were also conducted. This was based on observations made of dying rainbow trout which implicate neurological or neuromuscular disorder due to increased internal copper concentrations. At concentrations near the Incipient LC50, mortality may continue for 8-9 days. Prior to death, many fish exhibited rapid spasmodic movements, loss of equilibrium, and ultimately convulsed for several minutes before dying. This behavior was also observed by Baker (1969) during his investigations of the

toxicity of copper to the winter flounder (<u>Pseudopleuronectes</u> <u>americanus</u>). He subsequently suggested that exposure to copper causes neurological disorders as indicated by the symptoms of Wilson's Disease. Baker's (1969) histological observations indicated additional symptoms of Wilson's Disease including necrosis of the kidney and destruction of hemopoetic tissue.

With this in mind a fifth objective was to study the effect of Cu on acetylcholine (ACh) induced contraction of the frog (<u>Rana</u> <u>pipiens</u>) rectus abdominus muscle. The objective of this experiment was to determine whether copper could have a direct effect on muscle activity. This was to test the hypothesis that the toxic action copper at least at threshold concentrations is internal by acting on the neuromuscular system.

In summary, in the present study, various aspects of copper toxicity were studied with the following objectives in mind.

1. To define the relative importance of hardness and alkalinity in modifying copper toxicity.

2. To investigate the toxic interaction of copper and acid pH.

3. To test the ability of rainbow trout to acclimate to copper and to acid using death as the indicating parameter.

4. To determine the effect of copper exposure and acid exposure on mucous secretion rates and to examine the effect of secreted mucus on the speciation of copper and on pH levels of the test water.

5.　　To determine the effect of copper on muscle contraction.

6.　　To evaluate these results on the basis of how they relate to the establishment of water quality criteria for copper and acid.

MATERIALS AND METHODS

Fingerling rainbow trout weighing 7-12 grams were obtained from Sam Livingston Fish Hatchery, Calgary, Alberta where they were maintained at 12 ± 1° C in partially recirculated well water. These fish were obtained in April, 1977 and were used throughout all of the experiments.

A small lot of rainbow trout was obtained from Forest Hills Trout Farm, Bluffton, Alberta where they were maintained in artesian well water which remains at $3-4^o$ C throughout the year. These fish were used for one toxicity test in the acclimation experiments at which time they were 9 months old and weighed 6-12 grams.

The fish obtained from Sam Livingston Fish Hatchery had been hatched and raised in water with similar background levels of heavy metals as the Department of Zoology aquatic facility water. Many of these fish were initially placed in the Department of Zoology aquatic facility water at 13 ± 1° C and near natural photoperiod. This was City of Edmonton water originally drawn from the North Saskatchewan River. This water was dechlorinated by charcoal filtration and the addition of sodium thiosulfate and the pH was lowered to 7.4 with muriatic acid. Background levels of copper in this water were measured at 2-5 ug/l during this study. Throughout the study these fish were transferred to holding tanks in the experimental lab to maintain a minimum supply of 200 fish, which were acclimated for

at least 2 weeks to a 10L-14D photoperiod and $13 \pm 1^\circ$ C water.
These holding tanks and the toxicity testing chambers were
continually supplied with artificial fresh water. The fish
were allowed to acclimate to the various water chemical condi-
tions for at least 7 days before being tested. All fish were
fed commercially obtained Ewos Trout Food daily supplemented
once weekly with fresh chopped beef heart. Twelve fish were
used in each concentration of toxicant and they were placed in
the testing chambers 3 days before introduction of the toxicant
began. Although feeding dropped markedly or even ceased upon
exposure to copper, the fish were offered food daily. Uneaten
food and other debris was siphoned from the testing chambers
daily.

To maximize the use of all the dosing units in obtaining
the dose response curves, all eight testing chambers were used
for adding the toxicant. Therefore, 12 fish were isolated by a
nylon screen in the holding tank prior to each toxicity test to
serve as a control group. Throughout all of the experiments,
only 1 control fish died.

Water Quality and the Baseline LC50 for Copper and Acid

A flow-through system, using artificial fresh water made up
from distilled steam condensate which was then passed through a
high capacity deionizing resin bed filter (Barnstead No. D8901)
was used in all tests except for one LC50 conducted with the
dechlorinated tap water.

Stock solutions of $CaCl_2$ (U.S.P. Grade), $NaHCO_3$ (as baking
soda) and $KHCO_3$ (Reagent Grade) were added to the deionized

water via a proportional diluter modified from Mount and Brungs (1967) to make up an artificial fresh water with a calcium hardness of 50 mg/l (as $CaCO_3$), alkalinity of 28 mg/l as ($CaCO_3$) and pH of 7.3. This water was mixed and cooled to $13^\circ \pm 1^\circ$ C and siphoned to each of eight glass testing chambers of 23 liters. Four of these were furnished with a proportional diluter modified from Brungs et al. (1967) and four with a doser modified from McAllister et al. (1972). The 90 percent particle turnover time (Sprague, 1969) was approximately 4 hours. All tanks were well aerated to ensure oxygen saturation. Crystalline reagent grade $CuSO_4$ was used for all copper toxicity tests and reagent grade H_2SO_4 was used for the acid pH experiments. Because of the inherent buffering capacity due to the presence of bicarbonates, approximately 20 hours were required for the nominal pH to be reached in the acid experiments. CO_2 levels never exceeded 5 mg/l and thus were considered inconsequential to the results.

The incipient LC50 (defined as that concentration which will kill 50 percent of the sample population while the remaining 50 percent live for an indefinite period of time) was determined by running all tests for at least 15 days. This was considered a reasonable amount of time as all mortality had ceased by the 9th day for copper exposure and the 12th day for acid exposed fish.

The results of these toxicity tests are henceforth designated as the baseline ILC50 values as all the other toxicity tests were based upon and compared with these results.

Copper and Acid Interaction

The interaction of copper and acid was studied using the method of Sprague (1969). The baseline ILC50 values for copper and acid were given a value of 1 toxic unit. Thus, the toxicity of various combinations or fractions of the lethal concentrations were tested.

Although the reduction of pH obviously reduced the alkalinity, the fish were acclimated to water consisting of the same hardness, alkalinity, and pH as in the baseline ILC50. Concentrated sulfuric acid was added with the copper in the Mariotte bottles to reduce the pH in each individual test chamber.

Two testing chambers were held at one pH value with copper concentrations being different in each chamber. Thus, 4 combinations of acid pH and a low and high copper concentration were tested simultaneously. The ILC50 was determined by comparing the mortality between the two copper concentrations at each nominal pH value. Two trials were required at each pH value and therefore, all four copper concentrations used at a particular pH value were plotted in the dose-mortality curve for calculating the ILC50.

Determining the Relative Importance of Hardness and Alkalinity

This experiment consisted of 3 separate sets of toxicity tests. These tests are outlined as follows:

Set 1

    a.   Low alkalinity   Low hardness

        (10 mg/1)   (12 mg/1)

    b.   Low alkalinity   High hardness

        (10 mg/1)   (100 mg/1)

Set 2.

    a.   Intermediate alkalinity   Intermediate hardness

        (28 mg/1)   (50 mg/1)

    b.   Intermediate alkalinity   High hardness

        (28 mg/1)   (100 mg/1)

Set 3.

    a.   High alkalinity   Low hardness

        (50 mg/1)   (12 mg/1)

    b.   High alkalinity   High Hardness

        (50 mg/1)   (100 mg/1)

Each set was run at one time. All modifications in alkalinity were obtained by varying the concentration of bicarbonate in the initial dilution of the deionized water. The lower hardness values in each set were obtained by changing the concentration of $CaCl_2$ in this manner while the higher hardness concentrations were obtained by adding the $CaCl_2$ with the copper solution in the Mariotte bottles for each testing chamber. In this way the effect of low versus high hardness values could be tested simultaneously.

Acclimation to Copper and Acid

The ability of rainbow trout to acclimate to copper was tested on fish from the same brood stock which had been pre-

viously exposed to three different sublethal concentrations of copper. (1) The fish obtained from Forest Hills Trout Farm were hatched and raised in heavy metal-free artesian well water. After transport to the University of Alberta's aquatic facility they were held in the aquatic facility water for approximately 8 days during which the temperature was gradually raised from 3.5° C to 13° C. At this time they were placed in the artificial fresh water. They were held under these conditions for approximately 4 weeks before being tested. The tap water had a normal background concentration of copper of 2-5 ug/l (.05-.1 toxic unit). Therefore, the 8 days of exposure to the tap water was the only exposure to heavy metals that these fish had experienced. (2) The fish obtained from Sam Livingston Hatchery had been hatched and raised in water with similar background levels of heavy metals as the University of Alberta's tap water (Sam Livingston Fish Hatchery personnel, personal communication) and were held in the tap water prior to the minimum 2 week acclimation period in the artificial fresh water. These were the fish used in determining the baseline ILC50. (3) Some of the fish from the Sam Livingston Fish Hatchery were allowed to acclimate to 20-30 ug/l (.4-.6 toxic unit) of copper in the artificial fresh water for 7 days. Their tolerance to copper was then tested by increasing the copper concentration to a level which caused mortality.

At the same time as the third group was being tested, some fish were also tested for their ability to acclimate to acid pH. In this experiment fish from Sam Livingston hatchery were al-

lowed to acclimate for 7 days to pH 4.9-5.1 (approximately .2 toxic unit) before reducing the pH to lethal levels.

Additional Toxicity Tests

Because the ILC50 values in this study are much lower than values reported in the literature an ILC50 was run with dechlorinated tap water used in the aquatic holding facilities. This water had calcium hardness, alkalinity and pH values similar to the artificial water used in determining the baseline ILC50.

Because the experiments outlined above required nearly 9 months to complete, an additional ILC50 was conducted after the experiments were done using the same water chemical conditions as in the initial baseline ILC50. This tested the validity of the assumption that the sensitivity of fingerling rainbow trout to copper did not change during the 9 months of growth and aging. The fish weighed 16-27 g at the time this experiment was conducted.

Analysis of the Toxicity Tests

Hardness and alkalinity were measured at least twice weekly or every time the stock solutions were replenished using the methods of A.P.H.A. (1975). The pH was measured with a Fisher 520 digital pH/mV meter at least 10 times per day during the baseline acid ILC50 and during the copper-acid interaction experiments to ensure that nominal pH values were maintained. These values were maintained within a range of $\pm$ .07 pH units for at least 97% of the exposure time. The pH was measured

every second day during the copper toxicity tests. Copper
concentrations were measured with an atomic absorption spectro-
photometer using the solvent extraction method of Traversey
(1971). Copper was measured at least twice weekly or every time
the stock solutions in the Mariotte bottles were replenished.
At nominal concentrations of 10-50 ug/l the measured copper
concentration never differed from the nominal value by more than
4 ug/l and at nominal concentrations of 50-120 ug/l the measured
copper concentration never differed from the nominal value by
more than 9 ug/l. Phosphates were measured 3 times during the
toxicity test with tap water and 3 times during the repeat ILC50
in artificial water according to the methods of A.P.H.A. (1975).

The dose-mortality curves of all toxicity tests were sta-
tistically analyzed using the method of Litchfield and Wilcoxon
(1949) to place 95% confidence limits and slope functions on all
ILC50 values. The detailed results of all of these analyses are
reported in the appendix.

Mucus

The rate of mucous secretion in response to exposure to
static solutions of copper and acid was tested. Each of eight
fish weighing 80-100 g($\bar{x}$ = 92 g) were allowed to acclimate for
10 days to a small aquarium containing 2 liters of water at 9° C
Water chemical conditions were identical to those used in the
baseline ILC50. During this time about 2/3 of the water was
replaced each day. The fish were fed once each day until the

12th day. This was to reduce fecal excretion into the test tanks and yet test the fish when they are still in a positive energy balance and not relying on body stores of energy.

On the 15th day, the water in each aquarium was replaced 3 times to remove the residual mucus and debris. Three fish were then exposed to 1.8 toxic units of copper (85 ug/l), 3 were exposed to 1.6 toxic units of acid (pH 4.0) and 2 fish held in the standard artifical fresh water (pH 7.3) served as controls. The values of 85 ug/l copper and a pH of 4.0 were chosen on the basis that they should be equally toxic; each caused approximately 75% mortality in 48 hours of exposure in the toxicity tests. This is an important factor as the exposure was only continued for 8 hours.

The nominal pH value was obtained by using acidified water for the replacement. This water had been reduced to pH 3.6 and had been well aerated for about one hour to remove excess CO_2. Copper was administered in 2 doses about 5 minutes apart to approximate the rate of exposure to the acid pH.

After the 8 hour exposure period, the fish were removed, any fecal pellets were siphoned off, and the entire volume of water in each tank was homogenized in a Waring blender to suspend all mucous particles. A 100 ml aliquot was then dialized against carbowax to reduce the volume to approximately 15 ml. The samples were then freeze-dried for approximately 15 hours for complete dryness and weighed.

This entire experiment was repeated using 170 ug/l copper (3.6 toxic units) and pH 3.8 (4 toxic units). However, in this second experiment the trout were allowed to acclimatize to the small aquarium for 21 days before being tested. This data was statistically analyzed using the Mann-Whittney U Test.

The ability of mucus to bind copper was determined titrimetrically. In this experiment a known concentration of mucus was titrated against a standard copper solution. Each of 3 fish was held in the 2-liter chambers and exposed to a pH of approximately 5.0 to enhance mucous secretion. After approximately 24 hours the fish were removed. The entire volume of water was homogenized and an aliquot of this solution was used as the titrant. A standard calibration curve was first prepared by adding known amounts of copper to a mucus-free sample of the test water to insure the proper performance of the Orion model 92 specific-ion electrode. When a concentration of 60-70 ug/l total Cu was reached this same standard was back titrated with the solution of mucus. A reduction in the mV reading was interpreted as a reduction in the concentration of cupric ion. Mucous samples from each of the 3 fish were tested independently.

The ability of mucus to chelate copper at reduced pH values was tested by reducing the pH of the standard and the mucous solution to the desired values. This eliminated the variability in ionic activity due to pH changes when the titrations were being conducted.

The ability of mucus to bind hydrogen ions was determined by exposing each of 3 fish to pH 4.0 in the 2-liter chambers and measuring the pH at 2 hour intervals throughout an 8 hour exposure period.

A Possible Mode of Toxicity

An experiment was designed to test the possibility of an internal mechanism of copper toxicity. In this experiment the rectus abdominus muscle of the frog Rana pipiens was removed and attached to a muscle transducer connected to an electrophysiograph. The muscle was continuously bathed in amphibian Ringer's solution.

Contractions were caused by adding a Ringer's solution which contained 10^{-5} M ACh. The ACh-Ringer's was first administered to ensure that the muscle was active and to calibrate the physiograph. The bath was then rinsed with normal Ringer's and replaced by a Ringer's containing 10^{-5} M copper. The muscle was held in this solution for about 20 minutes at which time a solution of 10^{-5} M copper, 10^{-5} M ACh and Ringer's was administered. This solution was alternately replaced with the ACh-Ringer's solution at about 5 minute intervals. This procedure was repeated with a rectus abdominus muscle from a second frog.

RESULTS

The initial ILC50 for copper, the replicate at the end of 9 months of experiments and the toxicity test with tap water are shown in Table 1. There is no significant difference between the values in the artificial freshwater but the ILC50 for copper in tap water was significantly higher ($p < 0.05$) than that in the artificial freshwater. The tap water contained 1.2 mg/l of thiosulfate used for dechlorination and approximately 0.15 mg/l of orthophosphate. The artificial freshwater contained less than 0.03 mg/l of orthophosphate and probably no thiosulfate.

Copper-acid interaction

The ILC50 values for copper and pH were 48 ug/l and 4.39 pH units (4.07×10^{-5} M H^+) respectively. These values were used to calculate the various fractions of a toxic unit for the copper-acid interaction experiment. Five individual ILC50 values for various combinations of copper and low pH were obtained to generate Figure 1. The interaction was more than additive at pH values of 4.7 (2×10^{-5} M H^+) and less. And in the presence of 0.5 toxic unit of copper, 1.1 toxic units of acid was required for an ILC50 indicating that there was a slight antagonistic effect under these conditions.

At a pH of 5.4 (3.98×10^{-5} M H^+) (0.1 toxic unit) there was a highly synergistic or potentiated effect so that 0.1 toxic units of acid and 0.4 toxic unit of copper was an incipient lethal condition for 50% mortality. This test was repeated with 4 concentrations of copper at pH 5.4 yielding an ILC50 value for copper of 22 ug/l.

Table 1. A comparison of the ILC50 values for copper obtained from an initial toxicity test and the replicate test conducted 9 months later both using freshwater of completely defined composition with the ILC50 obtained using dechlorinated tap water. The slope function (S) is an expression of the dose response curve and is presented here for comparison to other dose response data.

	ILC50 for copper ug/l**	S**	pH*	water characteristics hardness mg/l* (as $CaCO_3$)	alkalinity mg/l* (as $CaCO_3$)
Initial	48 (42-54)	1.36 (1.2-1.6)	7.2 (7.1-7.3)	49 (46-54)	28 (24-31)
Final	46 (37-53)	1.5 (1.48-1.54)	7.2 (7.1-7.3)	51 (49-55)	30 (27-32)
Tap water	63 55-72)	1.4 (1.2-1.6)	7.3 (7.2-7.4)	57 (45-74)	33 (25-39)

*   parentheses enclose entire range

**   parentheses enclose 95 % confidence limits

Figure 1. The toxic interaction of acid pH and copper. Each

point represents an ILC50 for copper at that partic-

ular value for pH. The dashed line represents the

linear addition of the toxic effect. The line fit

to the four points above the additive line indicates

the linear relationship where acid is stressing the

fish (see text for explanation). The point at pH 4.35

and 14 ug/1 copper suggests an antagonistic interac-

tion while the point at pH 5.4 is definitely non-lin-

ear with these points and indicates a synergistic in-

teraction. The baseline ILC50 was determined at pH

2.3
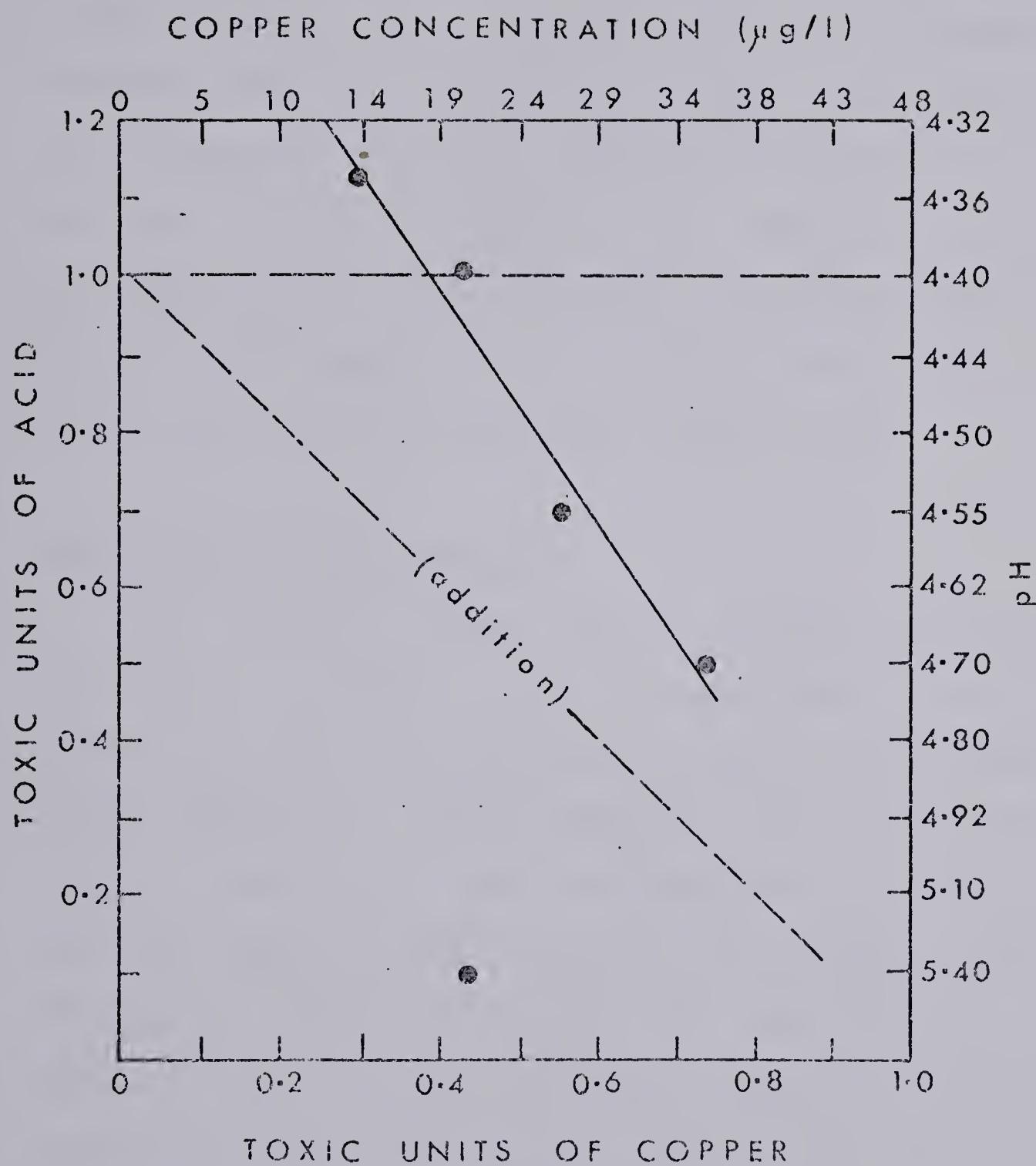
Hardness and alkalinity experiment

The results of the toxicity tests with various combinations of hardness and alkalinity are shown in table 2, this relationship is further illustrated in Figure 2. It is apparent that calcium hardness is more important than alkalinity in reducing copper toxicity. At a hardness of 12 mg/l, increasing the alkalinity from 10 to 50 mg/l had no effect on the ILC50 while at an alkalinity of 10 mg/l, increasing the hardness from 12 to 100 mg/l increased the ILC50 by 3-fold. However, at a hardness of approximately 100 mg/l increasing the alkalinity from 10 to 28 mg/l reduced copper toxicity by 44% and increasing the alkalinity from 28 to 51 reduced copper toxicity by 23%.

Acclimation to copper and acid

The ability of rainbow trout to acclimate to copper is illustrated in Figure 3. The fish that were obtained from Forest Hills Trout Farm which had not been previously exposed to copper exhibited the lowest ILC50 (28 ug/l) (95% confidence limits = 20-38 ug/l). This was significantly lower (p$<$0.05) than the baseline ILC50 (48 ug/l) (95% confidence limits = 40-55 ug/l) obtained with trout from Sam Livingston Fish Hatchery which had been previously exposed to a background concentration of approximately 5 ug/l Cu. This value was in turn significantly lower than the ILC50 (82 ug/l) (95% confidence limits = 69-93 ug/l) obtained with fish from Sam Livingston Fish Hatchery which had been acclimated for 7 days to 25-30 ug/l copper (0.5 toxic unit) prior to being tested.

Table 2. Effects of alkalinity and hardness of test water on the ILC50 of copper to fingerling rainbow trout. Alkalinity and hardness are expressed as mg/l CaCO$_3$.

Set	Alkalinity (mg/l)*	Calcium Hardness (mg/l)*	ILC50 (ug/l)**	pH*	S**
1. a.	10 (8-13)	12 (9-14)	19 (16-22)	7.1 (6.97-7.23)	1.36 (1.2-1.6)
b.	10 (8-13)	99 (92-105)	54 (46-61)	7.0 (6.91-7.09)	1.36 (1.2-1.6)
2. a.	28 (24-31)	49 (46-54)	48 (42-54)	7.3 (7.18-7.42)	1.41 (1.2-1.6)
b.	28 (24-31)	98 (92-107)	78 (70-86)	7.2 (7.05-7.35)	1.55 (1.03-2.3)
3. a.	51 (45-55)	12 (9-15)	18 (15-22)	7.4 (7.27-7.53)	1.56 (1.2-2.0)
b.	51 (45-55)	97 (93-104)	96 (87-108)	7.3 (7.2-7.4)	1.36 (0.96-2.2)

* Parentheses indicate the entire range of measurements.

** Parentheses indicate the 95% confidence limits.

Figure 2. The effect of various combinations of calcium hard-
ness and alkalinity (primarily as HCO_3^-) on the In-
cipient LC50 of copper to fingerling rainbow trout.
Error bars indicate the 95% confidence limits for
each Incipient LC50.
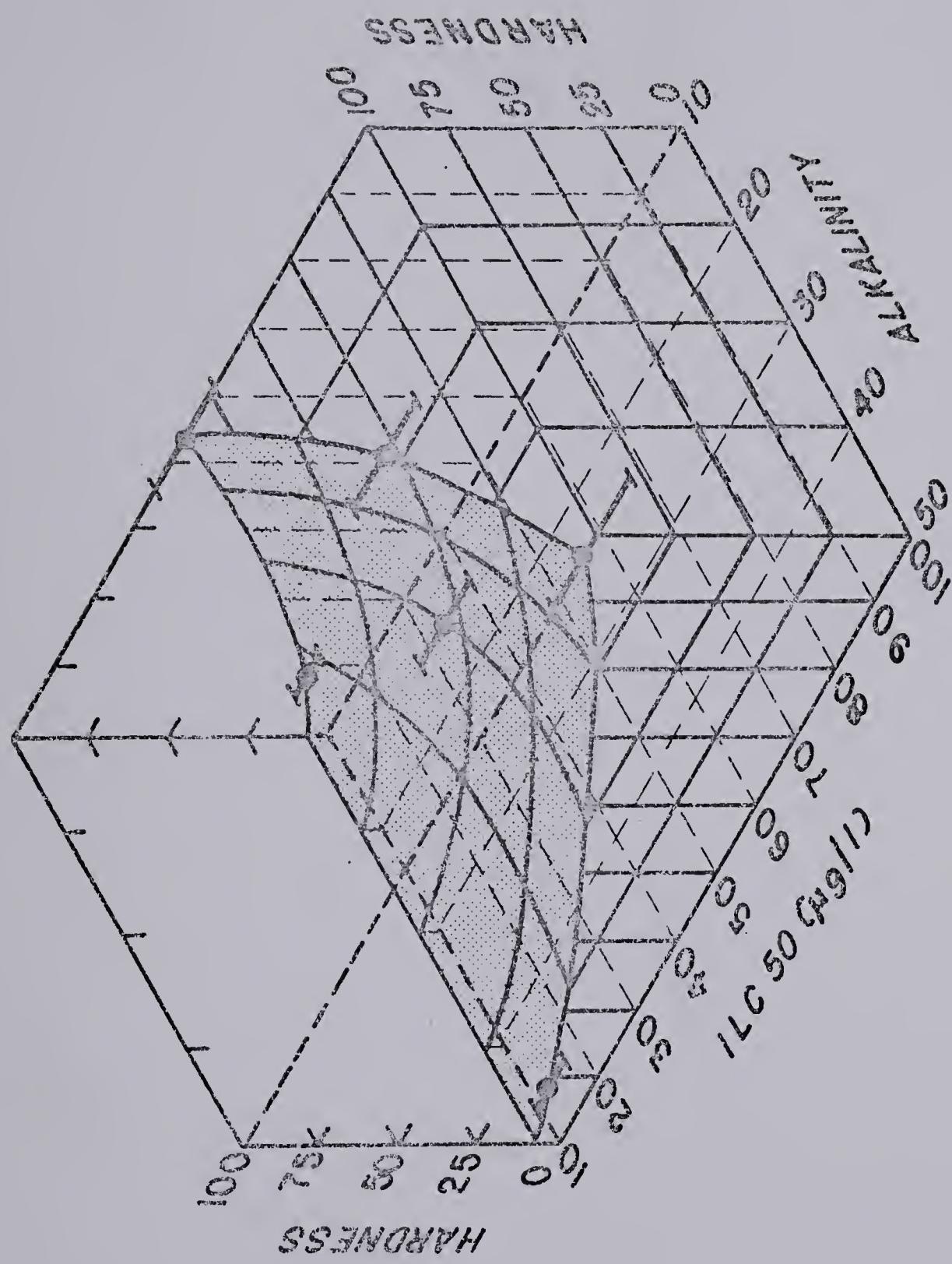
Figure 3. Mortality curves indicating the ability of rainbow

trout to acclimate to copper. All fish were obtained

from the same brood stock and were the same age

(10-12 months). One group of fish ($\triangle$) was raised

in heavy metal-free artesian well water. A second

group (O) was raised in water containing approximately

.1 TU and a third group ($\square$) was raised with the second

group but exposed to .5 TU for 1 week prior to the

toxicity test. Error bars indicate the 95 % confidence

limits.
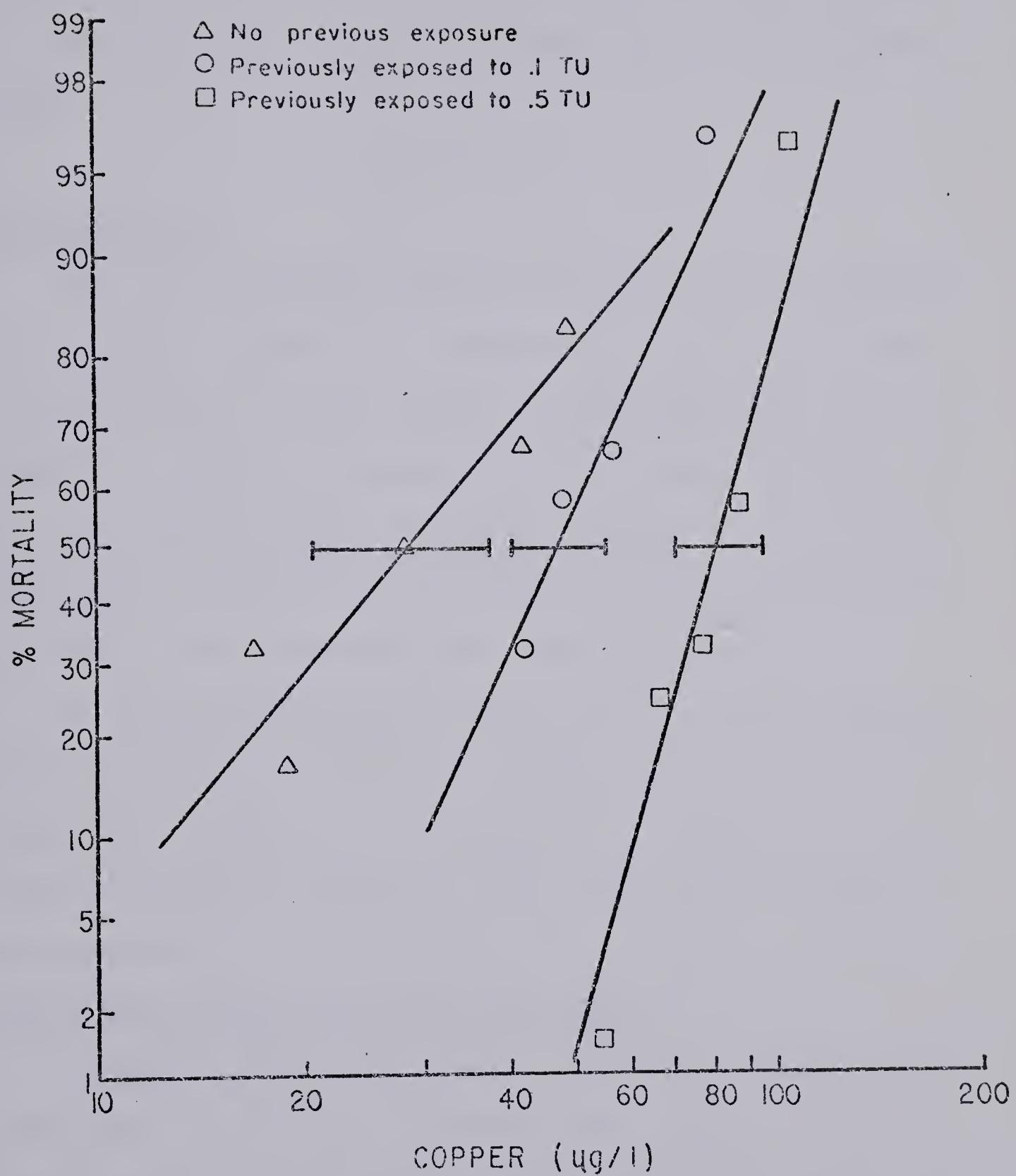
The baseline ILC50 for acid pH was 4.39 (4.0×10^{-5} M H^+). Exposure to sublethal levels of acid pH (4.9-5.1 or 0.2-0.3 toxic unit) for 7 days did not increase the ability of rainbow trout to tolerate previously toxic pH values (Figure 4). The ILC50 after sublethal exposure was a pH of 4.44 (3.63×10^{-5} M H^+) which was within the 95% confidence limits of the baseline ILC50.

Mucous secretion

The mucous secretory response to copper and acid exposure is summarized in Table 3. Exposure to acid caused a significantly greater ($p < 0.05$) amount of mucous secretion than did exposure to copper. In experiment I, acid caused an average of 41% more mucous secretion while in experiment II, where acclimation time was twice as long (21 days) there was 30% more mucus secreted by acid exposed fish than copper exposed fish.

It is also interesting to note that the average secretion in all treatments of experiment II was approximately 1/2 of the values for experiment I. In addition mucous secretion in response to copper was similar to that found for control fish in each experiment.

Copper chelation and acid buffering by mucus

A copper solution was titrated against 3 different concentrations of mucus from 3 different fish (figures 5, 6 and 7). These results clearly indicate that at pH 7.3 the addition of the mucous solution effectively reduced the cupric ion activity. Mucoprotein binding to the membrane of the electrode could not account for the decrease in Cupric ion activity (see appendix I).

Figure 4.   Mortality curves indicating the lack of ability of rainbow

trout to acclimate to acid pH.   One group of fish (□)

was raised and held in neutral pHs while a second group

(○) was held in pH 4.9-5.1 for one week prior to the

toxicity test.   Error bars indicate the 95 % confidence

limits.
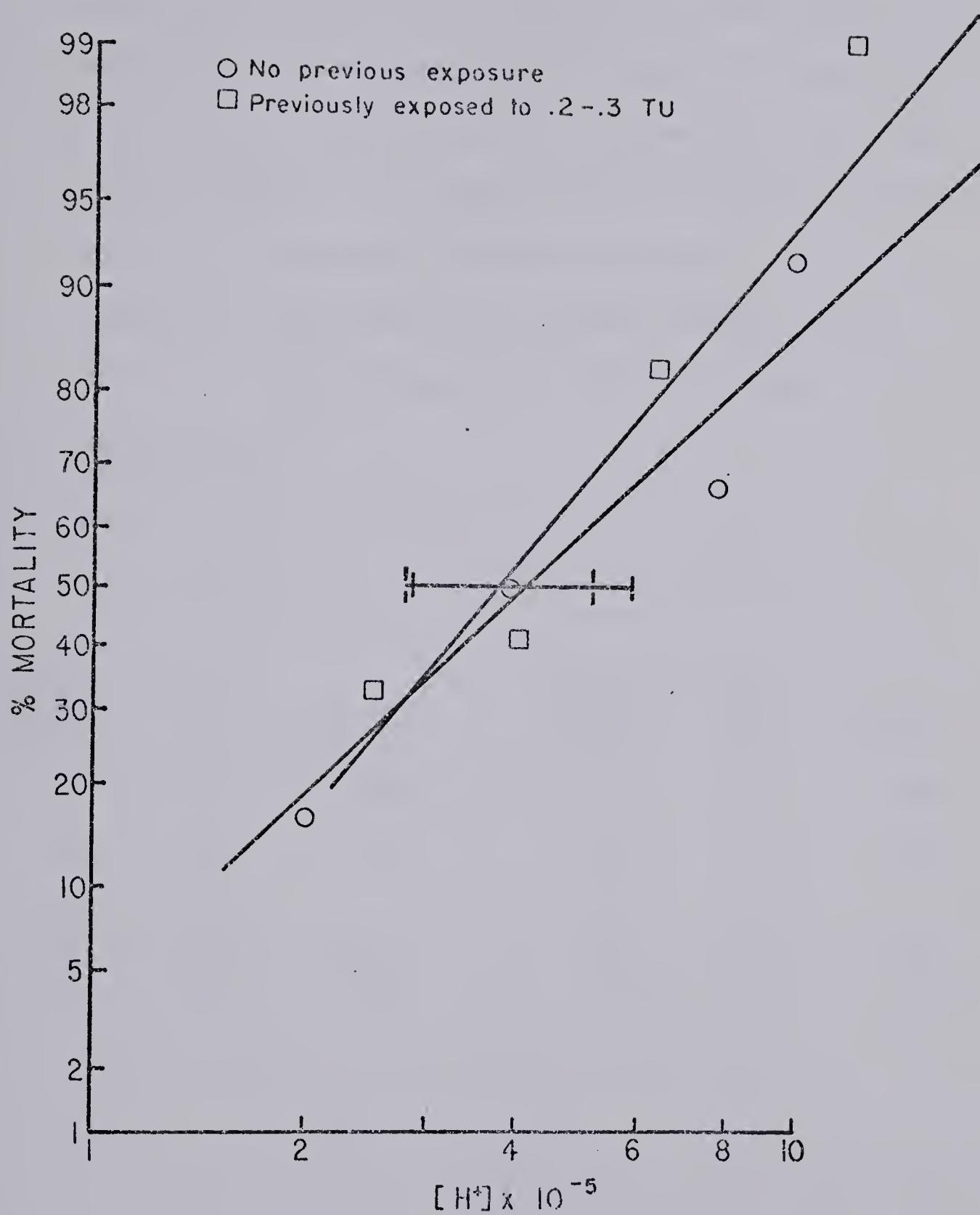
Table 3. The rate of mucous secretion (g per 8 hours) by rain-
bow trout when exposed to copper and acid pH. Each num-
ber is derived from an individual fish. In experiment
I the fish were allowed 10 days to acclimate while in
experiment II they were allowed 21 days to acclimate to
experimental conditions before the toxicants were added.
Values indicate total weight of mucus secreted in 8 hours
in 2 1 of test water. The Mann-Whittney U test indi-
cated that mucous secretion was significantly higher
($p < 0.05$) in acid exposure than copper exposure in both
experiments.

	I				II		
	85 ug/1 Copper	pH 4.0 Acid	Control		170 ug/1 Copper	pH 3.8 Acid	Control
	.550	.678	.395		.237	.311	.249
	.300	.616	.341		.282	.336	.309
	.608	.756			.259	.360	
$\bar{x}$	.486	.683	.368		.259	.336	.279

Later, because of the unexpected results of the copper-acid interaction this investigation was extended to test the copper-binding ability of mucus at various pH values. It can readily be seen that the ability of mucus to bind copper decreases as the pH is lowered. At near neutral pH (7.3), the addition of mucus rapidly reduced the cupric ion activity. At pH 3.5, large amounts of the mucous solution were required to reduce the cupric ion activity and at pH 3.0 the addition of mucus did not affect cupric ion activity.

Figure 8 illustrates the ability of rainbow trout to modify the pH of the ambient water. In 8 hours all fish were able to raise the pH from 4.0 to approximately 6.5. After approximately 24 hours the pH was very near neutral (approximately 6.95) indicating a continued but a reduced rate of increase above pH 6.5.

A possible internal mode of toxicity

The isolated frog rectus abdominus muscle was exposed to copper and ACh separately and in combination several times. Exposure to copper alone caused a slight but noticable con-traction (Figure 9) When copper was added with the ACh-Ringer's solution there was a greater contraction than when ACh-Ringers was used alone (Figure 10).

In addition after copper had been in the bathing medium for several minutes, the subsequent contraction caused by the addi-tion of the solution of copper, ACh and Ringers lead to large, rapid spasms or convulsions. (Figure 11). With the replacement

Figure 5. The influence of a solution of mucus (.01 g/100 ml)

obtained from a trout weighing approximately 90 g on

the ionic activity (mV) of copper solutions adjusted to

various pHs. For each curve a fresh copper solution of

50 ml containing 60-70 ug/l copper was used. These

solutions had a beginning reading of 100-120 mV. The

copper solution and mucous solution were adjusted to the

desired pH value. The mucus can reduce the ionic activity

of copper at pHs as low as 3.5 but at pH 3.0 the mucus

appears to be completely hydrolysed - or perhaps all

binding sites are occupied by H^{+}.
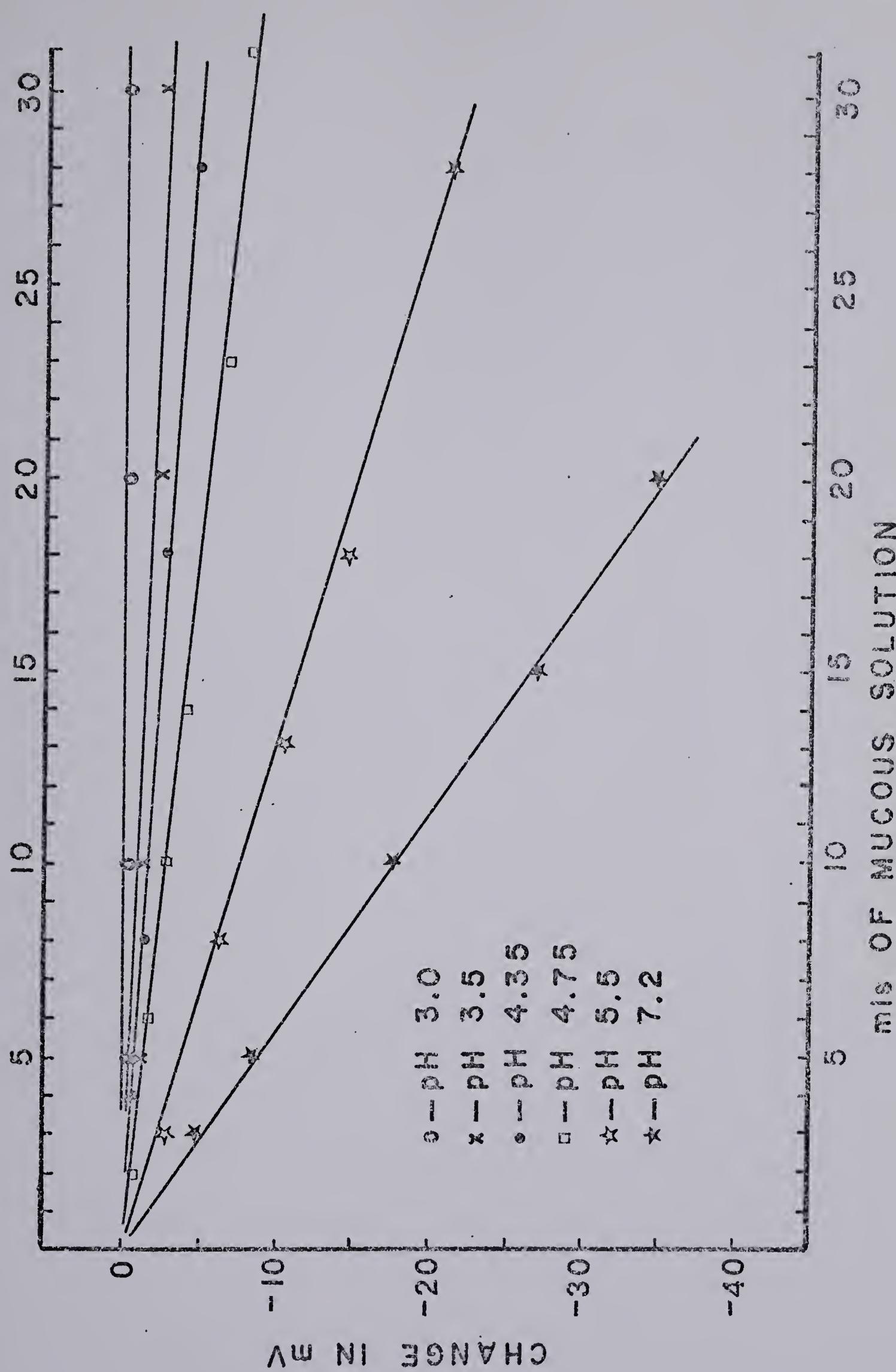
Figure 6. The influence of a solution of mucus (.04 g/100 ml)

obtained from a trout weighing approximately 85 g

on the ionic activity (mV) of copper solutions ad-

justed to various pHs. For each curve a fresh cop-

per solution of 50 ml containing 60-70 ug/l copper

was used. These solutions had a beginning mV read-

ing of 100-115. The copper solution and mucous solu-

tion was adjusted to the desired pH value.
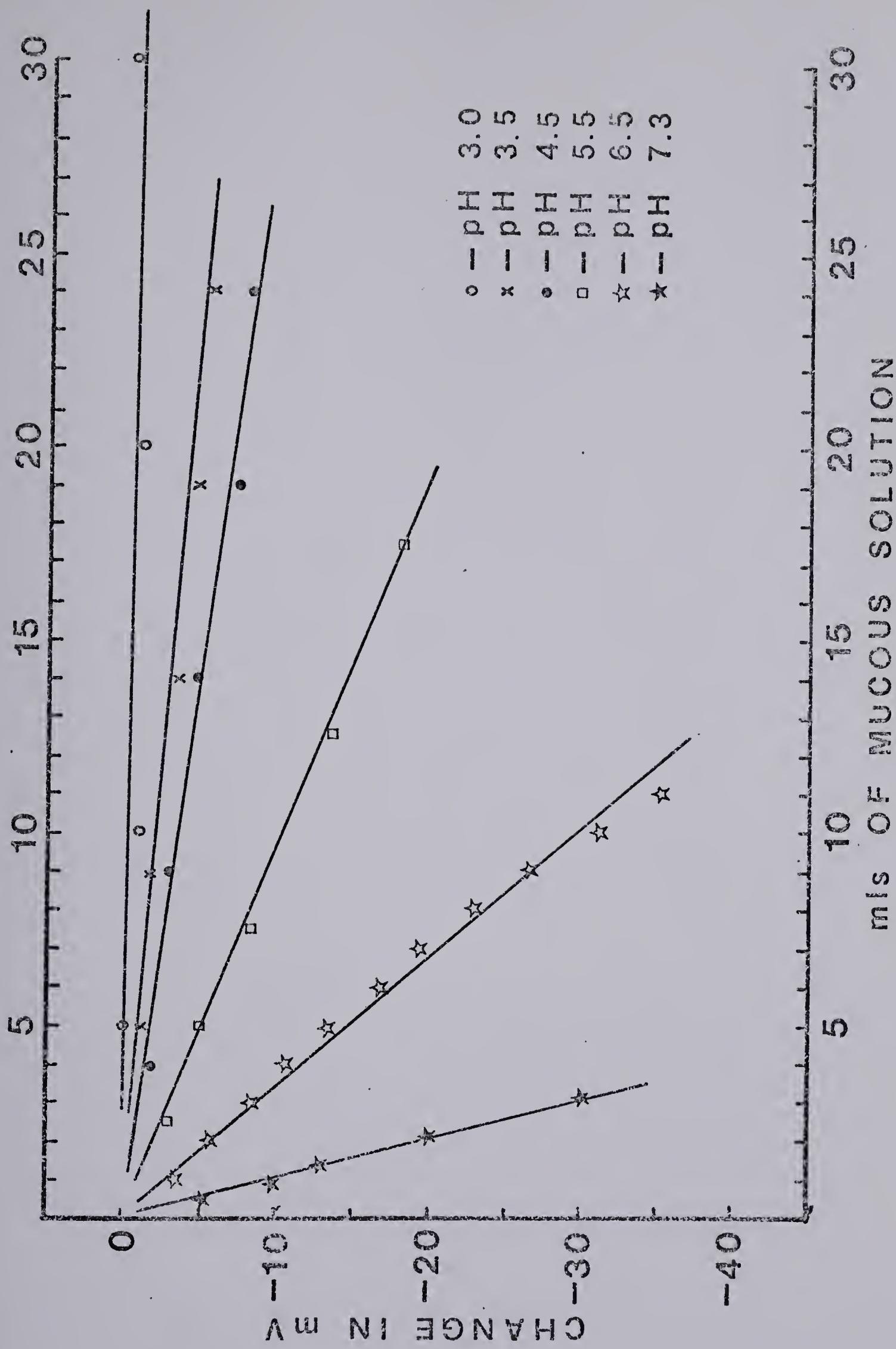
Figure 7. The influence of a solution of mucus (.035 g/100 ml)

obtained from a trout weighing approximately 90 g on

the ionic activity (mV) of copper solutions adjusted to

various pHs. For each curve a fresh copper solution of

50 ml containing 60-70 ug/l copper was used. These

solutions had a beginning mV reading of 105-115. The

copper solution and mucous solution was adjusted to

the desired pH value.
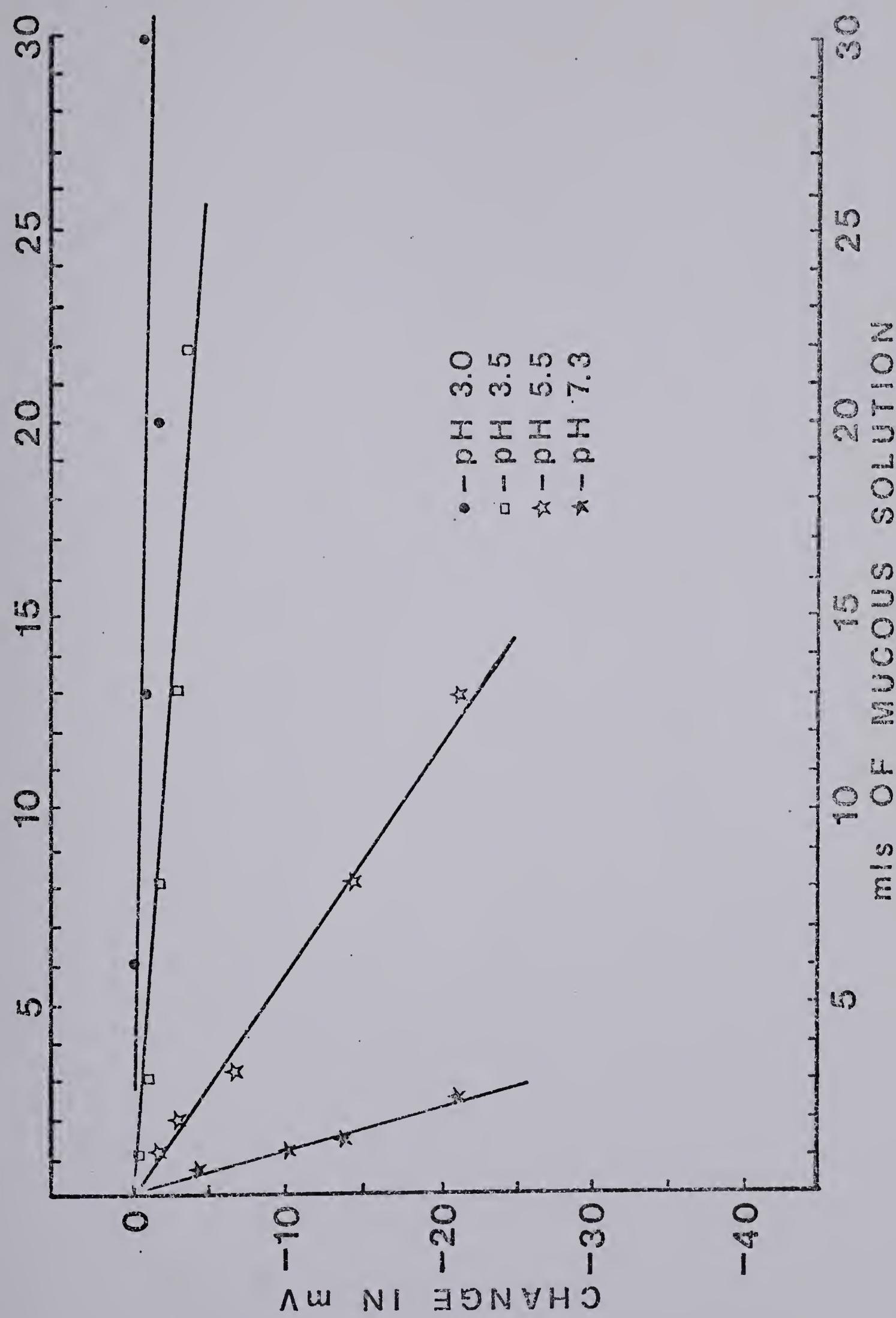
Figure 8.   The ability of rainbow trout to influence the ambient

pH.   Three 80-100 g trout were initially exposed to

pH 4.0 each in 2 liter tanks.   They could raise the pH

to nearly 7.0 after 8 hours which would be equivalent

to adding approximately 20 ml of .1 N NaOH. Error

bars indicate the standard error.
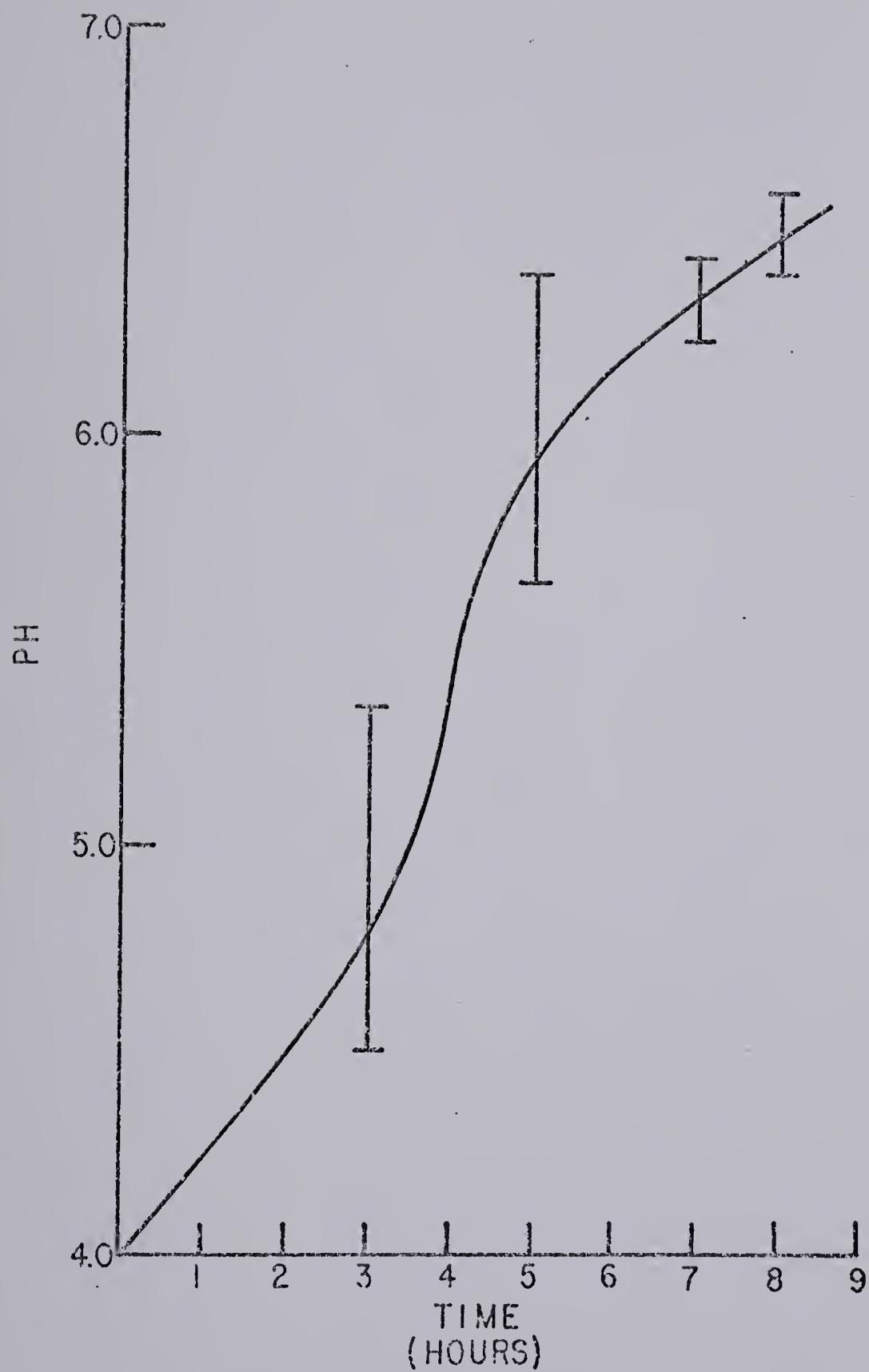
Figure 9. The response of isolated frog rectus abdominus muscle

to intermittent exposure to copper (10^{-6} M in amphibian

Ringer's solution) and to normal amphibian Ringer's

solution. Copper caused a slight and slowly developed

contraction.

TENSION

Normal
Ringer's

Copper

Normal
Ringer's

Copper

Copper

TIME
(minutes)
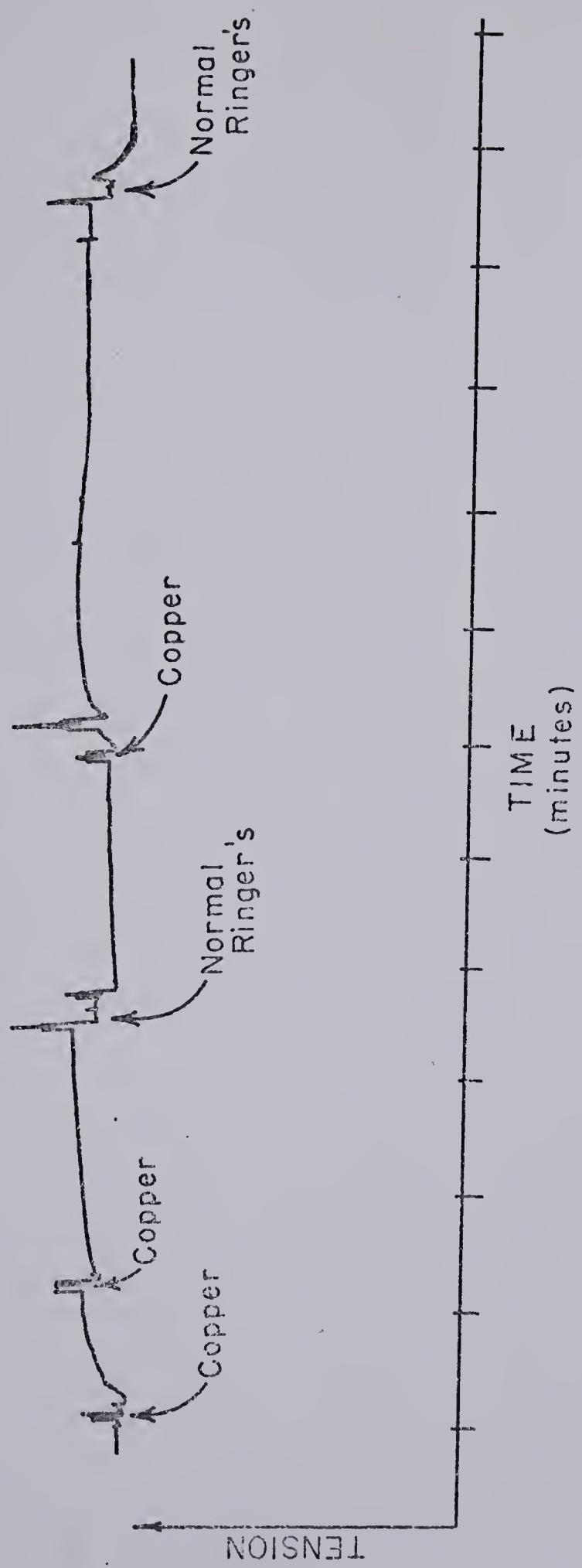
Figure 10. A comparison of an ACh (10^{-5} M) induced contraction

of isolated frog rectus abdominus muscle and a contraction

induced by a copper (10^{-6} M)-ACh solution after the

muscle was held for approximately 20 minutes in a solution

of copper and normal Ringer's. A greater contraction was

caused after copper was added to the system.
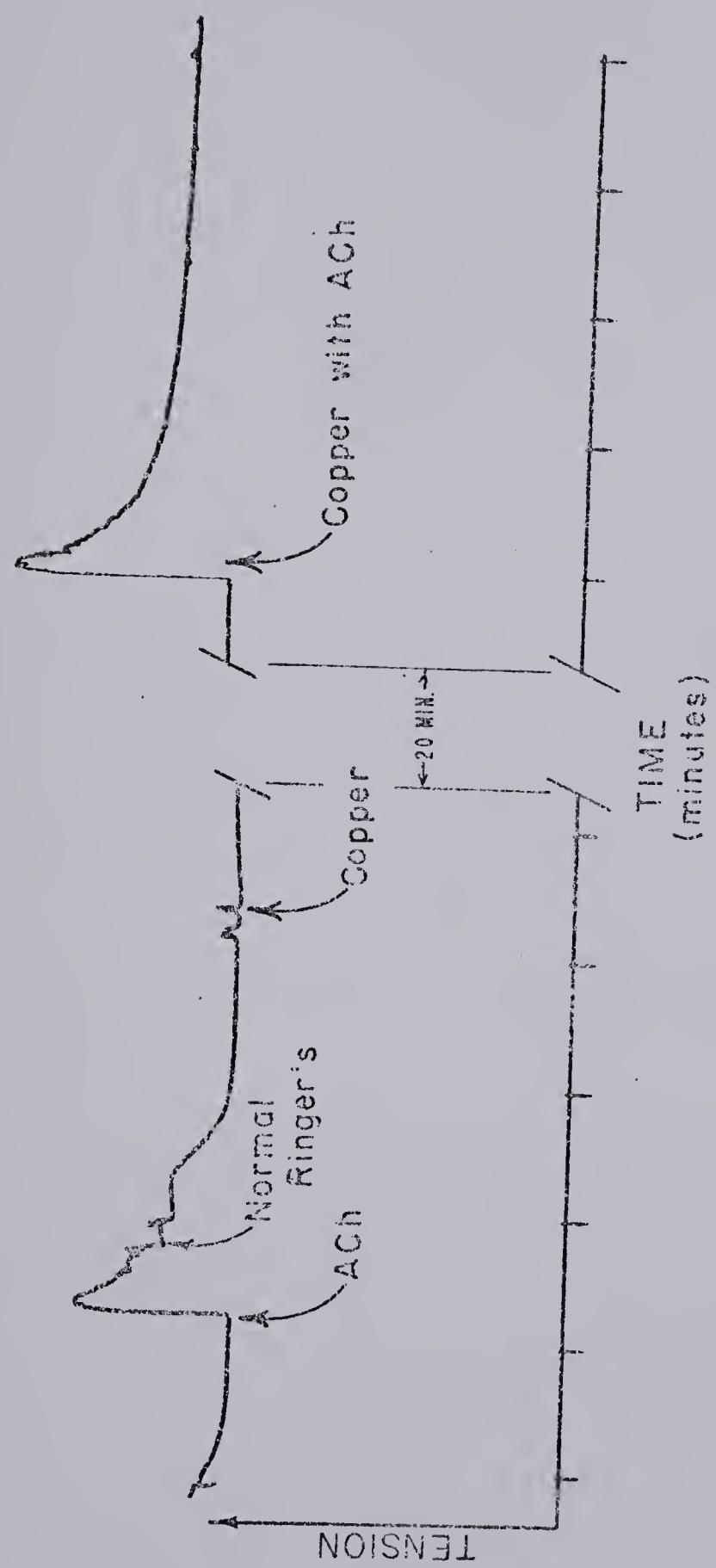
Figure 11.   The delayed response of isolated frog rectus abdominus

muscle to a solution of copper (10^{-6} M) and ACh (10^{-5} M)

The spontaneous spasms began approximately 12 minutes

after the solution was administered and continued until

the bathing medium was changed to a copper-free ACh

solution.

TENSION

Copper + ACh
12 minutes
previously

ACh with
no copper

TIME
(minutes)
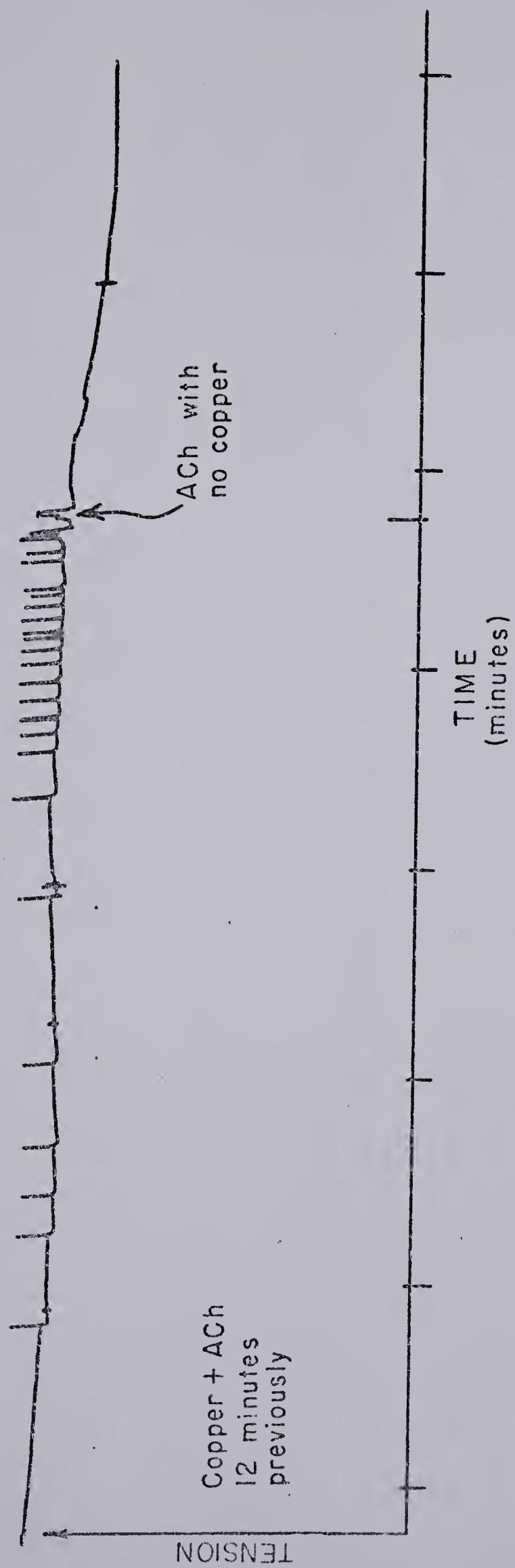
of this solution by an ACh-Ringer's solution the convulsive activity ceased but the contraction was maintained. When the solution of copper, ACh and Ringers was reintroduced the convulsive activity soon began once again as illustrated in Figure 12 which is a continuation of Figure 11.

Figure 12.   The response of the rectus abdominus muscle used in

Figure 11 following the reintroduction of the

copper-ACh solution.

Copper + ACh
7 minutes
previously

TENSION

TIME
(minutes)
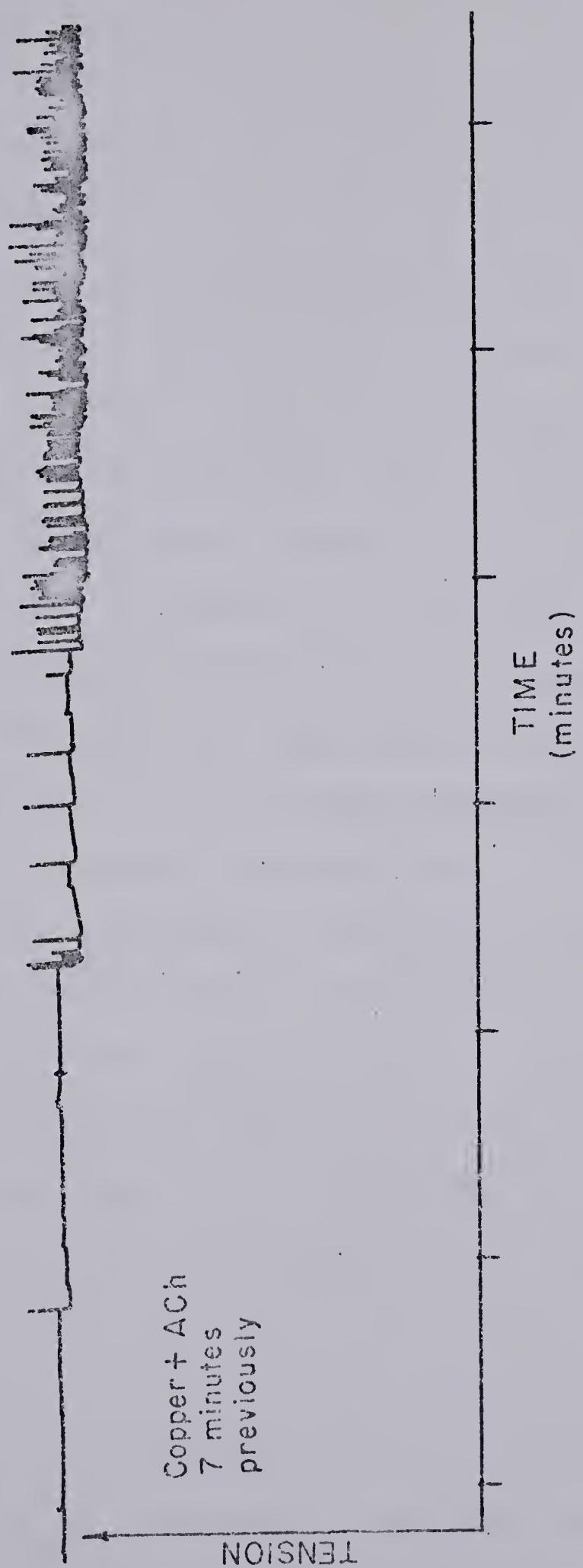
DISCUSSION

Copper and pH Interaction

The concept of the toxic unit originated with application to pollutants ranging from zero to lethal concentrations. Thus, the concept of toxic units to test the interaction of copper and acid may be controversial. This is brought out by the nature of the pH scale which precludes the use of a zero concentration of H^+. Therefore, in applying the concept of toxic units to this experiment the zero concentration or "no effect" concentration was assumed to be in the vicinity of pH 5.5. This seems acceptable in that mortality (Menendez, 1976), reduced weight gain (Jacobsen, 1977), or tissue damage (Daye and Garside, 1976) have only been noted at pH values of 5.2 or less. Therefore, it is with this assumption that the use of toxic units is applied to this experiment.

The less than additive interaction of copper and low pH (below 5.4) observed in the present study was entirely unexpected. It has been suggested that ionic copper (Cu^{++}) is the toxic species of copper (Stiff, 1971, a and b; Pagenkopf, et al. 1974; Shaw and Brown, 1974). The concentration of cupric ion is inversely related to the concentration of carbonates and hydroxides which in turn are dependent upon the pH of the water (Sylva, 1976). Sylva (1976) reported that the percentage of copper as Cu^{++} increases sigmoidally as pH values drop below 8.3 and there is no inorganically complexed copper at pH values below 5.5. In view of this information and the apparently

similar mechanisms of toxicity one would expect that the interation of copper and acid pH would be potentiated at all pH values below neutrality.

However, this was not the case at pH values below 5.4. At the hydrogen ion and copper concentrations tested here it is apparent that two separate mechanisms of interaction occurred. The ILC50 values expressed as the sum of the toxic units (TUs) for copper and pHs below 5.4 remains nearly the same (approximately 1.3 TU) while at pH 5.4 the sum of TU's was 0.6. Hence, at pH 5.4 copper and acid were highly synergistic, yielding an incipient LC50 with only 22 ug/l copper. However, as the pH was further reduced an antagonistic interaction occurred. For example, the same amount of copper (22 ug/l) was required to cause an incipient LC50 at pH 4.4 (1 TU of pH) as at pH 5.4 (0.1 TU of pH). It is unlikely that at pH 5.4, hydrogen ion was acting directly on the fish as Menendez (1976) reported that 5 months of exposure of brook trout to pH 5.5 caused no mortality. Similarly Jacobsen (1977) reported no reduction in weight gain of brown trout (Salmo trutta) exposed to pH 5.44 or 5.00 and Daye and Garside (1976) found that tissue damage only occurred after exposure to pH 5.2 and lower. Thus copper must have been the key factor in causing mortality at pH 5.4. It is concluded that the reduction in pH to 5.4 liberated cupric ion from all inorganic complexes in the water and thus greatly potentiated the toxicity of copper.

The less than additive effect of copper and acid at lower pH values may be due to a response evoked by acid which also

confers resistance to copper exposure. One explanation for such a response is that acid exposure stimulates greater mucous secretion than copper when fish are exposed to equally toxic concentrations of both (Table 3) and due to the protein component, this mucus is able to chelate copper even at pH 3.5 (Figures 5 through 7). Lau et al. (1974) studied a small peptide, diglycil-L-histidine, which could effectively bind copper at pH 4.6 and several authors have reported the ability of single amino acids to bind copper (Neumann and Sass-Kortsak, 1967; Stiff, 1971b; Sillen and Martell, 1964). The greater mucous secretion evoked by acid exposure and the tremendous ability of mucus to chelate copper even at very low pH values may be a key component in eliciting a more than additive response to the combinations of copper and acid.

Although different types of interaction of copper and acid pH were observed in this experiment the most important aspect of this experiment and those of Waiwood and Beamish (1979) and Howarth and Sprague (1978) is that any reduction in pH below neutrality can significantly increase copper toxicity.

Mucus Secretion in Response to Pollutants

The secretion of mucus in response to pollutants has long been considered a detrimental stress response causing death by suffocation. However, the results presented in this thesis indicate that mucus may be an important factor in combating the toxic effects of copper and acid pH.

The immediate rapid build-up of mucus in the testing chambers in response to copper or acid exposure in these toxicity tests, leading to the subsequent need for daily siphoning of mucus from the tanks indicates that mucus is shed freely into the water. In addition, this secretion continued for the duration of all toxicity tests. Therefore, it is not likely that there was extensive destruction of mucous cells throughout the exposure time. Rather, there was a continued production and secretion from the mucous cells. This was especially apparent in acid exposed fish (Table 3).

The presence of mucus in the medium apparently has an effect on cupric ion activity. During the determination of baseline ILC50's an attempt was made to measure cupric ion activity via a cupric ion specific electrode. A valid standard curve could be made at concentrations as low as 4 ug/l (10^{-8} M). Although an ionic strength adjuster made of a solution of $NaClO_4$ was added to make the background ionic activity high and consistent between standards and samples, the millivolt reading of samples taken from the testing chambers containing approximately 100 ug/l copper was lower than the blank standard. This indication of the low conductivity of the water is common in organic-rich natural water. In theory, taking into account the inorganic ions and pH of the water, approximately 10 ug/l cupric ion should have been detected.

Pickering (1976) analyzed brown trout mucus and found it to be about 1,000 parts protein to 6 parts carbohydrate. This lead

to the hypothesis that due to the protein component mucous se-
cretion could have a significant effect on the speciation of
copper and consequently on the toxicity of copper.

Figures 5, 6, and 7 show that mucus has a remarkable abili-
ty to chelate copper. At pH 7.3 there is a substantial reduc-
tion in the mV reading with very small additions of mucus. This
ability of mucus to bind Cu gradually decreases as the pH is
reduced until at pH 3.0 there are no reductions in mV with the
addition of mucus. Since copper displaces H^+ ions from the
peptide molecule in binding (Lau et al., 1974), there must be a
very strong affinity for Cu^{++} ions to allow a bond to form at
the low pH of 3.5.

Table 3 indicates that copper exposure did not enhance
mucous secretion above control levels. This may be related to
the observations of Labat et al. (1974) who found that copper
exposure caused an apparent immediate ejection of the mucous
cell contents followed by a possible decrease in mucous se-
cretion. Over a period of time this could result in no change
or even a net reduction in mucous secretion as indicated in
Experiment II of Table 3.

The only functional importance of this type of response may
be in combating a transient increase in copper concentrations in
the environment. The rapid secretion of mucus with its remark-
able ability to bind cupric ions (Figures 5-7) would serve as a
valuable defense for such an encounter with copper.

Experiment II (Table 3) was designed to reduce the effect
of handling stress on mucous secretion as well as to test the

effect of different concentrations of toxicants. The 14 day acclimation period prior to testing apparently was not enough to eliminate a substantial influence from handling stress. However, in Experiment II the mucous secretion in each treatment was approximately half of that in Experiment I - probably due to allowing a longer acclimation period.

It was noted that the low secretion rates in Table 3 agree with the observations of Labat et al. (1974). However, this contradicts the observations of continued mucous secretion in the toxicity tests. The apparent inhibition of mucus secretion may be the result of cell damage caused by extremely toxic copper concentrations. Labat et al. (1974) used concentrations of 1,000 and 1,500 ug/l (approximately 20 times the ILC50) and the mucus secretion experiments in the present study were conducted in 2 and 4 times the ILC50.

Contrary to the response elicited by copper exposure, acid pH significantly enhanced mucous secretion (Table 3). This could partly be the result of an apparent increase in the number of mucous cells in the gill lamellae upon exposure to acid pH (Plonka and Neff, 1969) or to a general stimulation of mucous production by acid conditions. The ability of mucus to buffer against acidic conditions (Figure 8) appears to be a valuable defense system against acid exposure the function of which would involve the presence of a minute buffering zone created by the secretion of mucus over the entire body.

The strong affinity of mucus for cupric and hydrogen ions together with the continual secretion of mucus would inhibit the

binding of the ions to the body and carry them away as the mucus is sloughed off. These experiments suggest that the secretion of mucus is not just a passive stress response, and consequently, an additional metabolic burden on the fish. It appears that the components and structure of mucus as a glycoprotein enable it to function as a highly efficient chelator and buffer against dissolved cationic poisons. Therefore, the ability to secrete excessive amounts of mucus upon exposure to certain dissolved toxicants appears to be beneficial to the animal.

Hardness and Alkalinity Experiments

The hardness and alkalinity experiments also yielded unexpected results in that current theory and recently published toxicity tests have related copper toxicity to alkalinity rather than to water hardness. This is based on the ability of many anions to complex copper and hence reduce the concentration of ionic copper (Andrew et al., 1977, Stiff, 1971b). However, there is some evidence indicating that non carbonated calcium salts (i.e. $CaCl_2$) may lead to reduction in copper toxicity (Jones, 1938; Ellis, 1937; Lloyd, 1965). Results of the present experiments (Figure 2) indicate that while bicarbonate alkalinity can protect against copper toxicity at high water hardness, calcium hardness is much more important than carbonate-bicarbonate alkalinity in protecting fish from copper. It appears that calcium must reach some threshold concentration between 12 and 28 mg/l before alkalinity begins to reduce copper toxicity. The ILC50 for copper was greatest when both the calcium and alkalinity levels were highest.

The significant reduction in toxicity due to increase water hardness may be due to competition of both these divalent cations for binding sites on specific proteins in the blood. In fact the transport of both copper and calcium as nutrients in the blood has been shown to be in binary or ternary combination with serum albumin (Lau et al., 1974; Sarker and Kruck, 1966; Katz and Klotz, 1953; Jacobs et al., 1971). Lloyd (1965) reported that calcium protection in rainbow trout continued for a few days after the fish were transferred to soft water. He thus concluded that the protective effect was internal, as it took several days for the calcium to 'leach out'.

However, there is evidence that calcium acts on membranes. Herrera and Curran (1963) and Meryman (1972) found calcium to inhibit the transport of sodium ions and suggested that calcium is bound to anionic sites in the membrane and decreases the permeability of positive ions. It seems likely that the release of calcium from these sites when a fish is placed in soft water could also require time and thus the protection may not be internal (Waiwood and Beamish, 1978). Because of their similar binding qualities, it is suggested that the non-toxic calcium competes with copper at sites on the external cellular membranes of the gills and inhibits the binding and transport of copper.

Acclimation to Acid pH and Copper

Acclimation may be an important defense mechanism against the toxicity of certain pollutants. Acclimation to acid pH was suspected because of: (1) The dynamic characteristics of the

blood bicarbonate buffer system whereby the fish might be able to better eliminate H^+ ions which enter the body by converting them to $H_2O + CO_2$; (2) The histological work of Plonka and Neff (1969) where they observed an increase in the number of mucous cells upon exposure to acid and (3) The findings in the present study demonstrating the ability of fish to buffer against acidic conditions (Figure 8).

However, the results of the acclimation experiments in the present study indicate either that the ILC50 of trout does not have the ability to acclimate to acid pH or that exposure to sublethal levels for one week was not long enough to elicit such a response. Previous exposure to pH 4.9-5.1 for 7 days did not increase the tolerance of rainbow trout to lethal levels of acid. Lloyd and Jordan (1964) also found that rainbow trout did not acclimate with previous exposure to pHs of 6.55, 7.5 and 8.4 for 5 days and Robinson et al. (1976) found that exposure of brook trout to pH 3.75 for 7 days only further reduced their tolerance to pHs of 2.5 and 3.0.

The mode of toxicity for acid has not yet been clearly defined. Blood PO_2 and pH of exposed fish have been monitored by several authors (Packer and Dunson, 1970; Eddy, 1976; Vaala and Mitchel, 1970; Dively et al., 1977). Packer and Dunson (1970 and 1972) and Vaala and Mitchel (1970) found that extremely toxic pH levels of 2.5-3.5 caused extensive mucification at the gills and significant reductions in blood PO_2 and pH. Packer and Dunson (1970) observed that there was no oxygen uptake for up to .5 hours prior to death in some of the fish

exposed to pH 3.25. However, Dively et al. (1977) exposed brook trout to near-threshold levels of pH for up to 5 days. Although several fish died in these tests blood PO_2 and pH in the remaining fish did not change significantly. But the visual symptoms of hypoxemic stress including gaping of the mouth, forced expansion of gill opercula, an increase in ventilatory rate, a marked reduction in swimming activity and extensive gill mucification were observed in these fish.

Dively et al. (1977) associated mucification with the metabolic state of the fish and subsequently with the tolerance of brook trout to acid exposure at various times of year. They observed that in January excessive mucous production was minimal in acid exposed brook trout. Coupled with this condition was an extended elevation in blood PO_2 and an increased rate and depth of ventilation which would tend to add additional oxygen to the body. However, in summer exposed fish greater mucus production was noted. This was accompanied by a transient drop in PO_2 and a higher rate of ventilation indicating hypoxemic stress. They attributed these results to the seasonal physiological and biochemical changes associated with increased nutrient uptake and growth which would result in a higher oxygen demand in summer. Thus any impairment of the blood-gas transport system during this time would result in a greater stress than at times when the oxygen demand was not as great. They concluded that this hypothesis adequately explained the direct causes of death in that no fish died in pH 4.2 in winter but 40% of the fish died in pH 4.2 in summer.

Thus, it appears that there may be a sensitive but definite threshold of tolerance for mucification and possibly gill damage. This threshold for gill damage may be dependent on the disintegration of mucous cells which seems to be related to the extrusion process. When the whole cell extrudes its contents the integrity of the contiguous squamous cells is disrupted (Plonka and Neff, 1969). This results in an increased folding of lamellae and often the interlocking of lamellae with those of adjacent filaments. Furthermore, densely staining nuclei were observed in the interlamellar spaces which, together with the extruded mucus, appeared to compose the coagulation film (Plonka and Neff, 1969). Consequently, if this coagulation film is extensive enough, oxygen transport would be inhibited and suffocation would occur (Plonka and Neff, 1969; Westfall, 1945, and Ellis, 1937).

The data obtained in the present study indicates that trout have a remarkable ability to acclimate to copper and this ability is demonstrated in a very short time. My data is supported by the observations of Paul (1952) and Grande (1967) who found that rainbow trout transplanted from heavy metal free water to water containing heavy metals could not survive while endemic populations of rainbow trout existed in the polluted water. Therefore, the transient elevation in ventilatory rate, oxygen consumption (O'Hara, 1971), several blood parameters (Christensen et al., 1972 and McKim et al., 1976) appear to be true indicators of the fishes ability to acclimate to copper. In the present study the ILC50 was nearly tripled (from 28 ug/l

to 82   g/1) between fish which had no previous exposure to copper and fish previously exposed to sublethal concentrations.

The physiological mechanism of acclimation to copper is not known but the sustained mucous accumulation during the toxicity tests observed in the present study suggests that it may involve an increase in the secretion of mucus from external surfaces and especially from the gills. Because of the efficient ability of mucus to bind copper (Figures 5 through 7), the mucous substances on the surface of the gills and that which has immediately been sloughed off the gills may bind ionic (Cu^{++}) copper and prevent it from binding to and entering epithelial cells of the gills.

However, there are two lines of evidence which do not support this theory. Baker (1969) reported that exposure of the winter flounder to copper actually reduced the number of distinguishable mucous cells while it increased the number of chloride cells. He noted that this change seems to be a conversion of mucous cells to chloride cells. This seems possible in that Munshi (1964) suggested that mucous cells can either secrete mucus or chloride ions and that mucous cells of freshwater fish can become functionally chloride-excreting cells when the fish are exposed to hypertonic salt solutions. Furthermore, as it is believed that chloride cells of freshwater fish take up chloride from the medium, salt loss may become another factor in copper toxicity. A significant reduction in blood Cl and osmolarity was indeed found to occur in brook trout exposed to near lethal levels of copper (McKim et al., 1970). Therefore, an increase

in the number of chloride cells may be an attempt by the fish to maintain electrolyte balance in the blood. Baker's observations support this suggestion, he noted that mucous cells are normally found on the lamellae while chloride cells were found in the basilamellar region. After exposure to copper the number of mucous cells was reduced and chloride cells were found in this region instead. Related to this, Labat et al. (1974), Pequignot et al. (1975) and Cusick (1967) exposed carp (Cyprinus carpio), trout (S. irideus) and the guppy (Poecilia reticulatas) respectively to copper. There was no mention of the effect on chloride cells. However, all reported an immediate and significant reduction in the number of stainable mucous cells. This was followed by an apparent reduction in mucous secretion.

Labat et al. (1974) carried their experiment one step further and found that when copper exposed fish were returned to clean water the mucous cells rapidly appeared again. Within 24 hours there was a significant increase in the number of stainable mucous cells. They concluded that copper caused an initial ejection of the contents of the mucous cells which resulted in the inability to distinguish these cells. Following this it was believed that copper reduced the ability to produce and secrete mucus. However, upon return to clean water, the same mucous cells appear to become active and functional again as indicated by the rapid return of distinguishable mucous cells.

Thus, it appears that there is an initial surge of mucous secretion as mucous cells eject their contents, this may be followed by an overall reduction in mucous secretion. The

observations on mucous secretion in the present study may support this hypothesis. In both experiments (Table 3) copper exposure did not cause secretion rates above control values and in the second experiment there was actually a reduction in mucous secretion below control values. However, on the other hand, these fish apparently initiated some immediate response which allowed them to survive these extremely toxic concentrations. Thus, it seems that the only response which could be initiated quick enough is the secretion of mucus from the body and gill surfaces.

A second line of evidence which does not support mucous secretion as a possible mechanism of acclimation to copper is that acid pH which has been observed to cause excessive mucous secretion from the body and gill surface (Daye and Garside, 1976; Vaala and Mitchell, 1970; Dively et al., 1977; Plonka and Neff, 1969) has also been found to cause an increase in the number of mucous cells (Plonka and Neff, 1969). This would seemingly increase the ability to secrete additional mucus. In addition, measurements of mucus secreted from the fish (Table 3) demonstrated that acid exposure greatly enhances mucous secretion. Yet with this apparent ability to increase mucous secretion and the remarkable ability of this substance to raise the pH (Figure 8). The ILC50 of rainbow trout did not acclimate to acidic conditions.

It therefore, seems likely that acclimation to the presence of copper in the medium water must be linked to some internal physiological phenomenon. The likely candidate responsible for

this process would be to increase the blood levels of ceruloplasmin, a copper-binding alpha-globulin protein manufactured in the liver and whose function is the binding and excretion of excessive amounts of copper in the blood (Peisach et al., 1967). As copper serves as a necessary micronutrient and must occur in trace concentrations, the levels of ceruloplasmin in the blood must undergo careful and dynamic regulation. Thus, it would be possible to manufacture and/or release ceruloplasmin in response to higher blood levels of copper. In addition, the manufacturing and release of this protein could seemingly take place immediately after copper enters the blood.

Mechanisms of Copper Toxicity

The resultant adverse effects of high concentrations of copper and low concentrations of ceruloplasmin in the blood have been studied extensively in the context of Wilson's Disease. It is the absence or low levels of ceruloplasmin in the blood which is the primary cause of Wilson's Disease (Peisach et al., 1967). In patients suffering from Wilson's Disease excessive copper accumulates in the brain, liver, kidney, hemopoetic tissue and the cornea. This toxic increase in copper results in hepatic necrosis, hemolytic anemia and deposition of copper in the cornea in the form of Kaiser-Fleischer Rings (Baker, 1969). These symptoms have ultimately been linked with the interference of copper with membrane ATPase (Peters, 1967) and the Lipoic-acid pyruvate system (Peisach et al., 1967), resulting in cirrhosis of the liver, destruction of brain neurons, necrosis of kidney tubules and in uncoordinated limb action and spontaneous jerky movements.

Several of these symptoms were identified in the winter flounder after exposure to near-threshold concentrations of copper (Baker, 1969). He reported fatty metamorphosis in the liver, necrosis in the kidney, destruction of hemopoetic tissue and spasmodic, uncoordinated movements. Similar histological damage of the kidney was also observed in the mummichog (Fundulus heteroclitus) exposed to 5000 ug/l copper (Gardner and LaRoche, 1973). Another important impairment found by Gardner and LaRoche (1973) was cellular changes in mechanoreceptors of the lateral line and chemoreceptor sites of the olfactory organs. Alteration in nervous transmission in the olfactory nerve was found in trout exposed to minute quantities, of copper (3-5 ug/l) (Hara et al., 1975). This could seemingly cause a reduction or elimination of the efficient use of these peripheral sensory systems. Although they did not make histological observations, Brungs et al. (1973) found that brown bullheads (Ictalurus nebulosis) accumulated copper in the liver, kidney, and gill and Vogel (1959) noted an extensive accumulation of copper in the kidney and central nervous system in goldfish.

Thus, there is evidence that copper indeed enters the body, accumulates in tissues which have a high metabolic rate and causes severe histological, behavioral and sensory alterations.

In the present study, jerky or spasmodic contractions similar to those described by Baker (1969) were observed in many of the fingerling trout just prior to death. These fish first exhibited difficulty in maintaining equilibrium which was accompained or immediately followed by rapid but most often single

propelling tail flips. This activity was followed by total loss of equilibrium with the fish assuming an inverted position and undergoing convulsive activity. These convulsions stopped just prior to the cessation of opercular movement.

This observation lead to the question of whether or not copper has a direct effect on neuromuscular systems and especially the muscles. Figures 10 through 12 illustrate the effect that copper has on isolated frog muscle. The convulsive activity demonstrated by the frog muscle is very similar to that observed in the trout.

It appears from these observations that copper may act in two ways; (1) Copper alone causes a slight contraction and when ACh is administered with copper there seems to be a somewhat greater contraction than if ACh is used alone (Figures 10 and 11). This indicates that there may be an immediate leakage of copper into the cell or possibly an active sequestering of copper because of its ability to mimic calcium; (2) after the muscle is bathed in the copper-Ringers solution, the subsequent contraction caused by the ACh-copper solution leads to rapid convulsive activity (Figure 12) indicating an impairment of the contractile mechanism or the action of the sarcoplasmic reticulum in sequestering Ca is impaired. These observations suggest some interesting and testable hypotheses which are discussed in Appendix V.

The frog muscle was exposed to concentrations nearly 10 times less than the observed increase in concentrations of copper in gill, kidney, and liver tissue of brown bullheads

(*Ictalurus nebulosus*) exposed to sublethal concentrations of copper (Brungs et al., 1973). It is conceivable then that copper could accumulate in muscle tissue to the extent that muscle activity is impaired and symptoms such as those exhibited by the dying fish and the frog muscle in these experiments are seen.

Thus, it seems possible that the toxic action of copper can be internal by causing liver and kidney damage and impairing sensory, nervous and muscular activity. However, it should be noted that in all of the toxicity experiments demonstrating such impairment (Hara, et al., 1976; Baker, 1969; Vogel, 1959; and the present study), fish were exposed to threshold or near-threshold concentrations for at least 7 days. At higher concentrations which cause a 48 or 96 hr LC50 it appears likely that the primary mode of toxicity is by causing gill damage, including separation of epithelial tissue, destruction of epithelial, mucous and chloride cells and possible subsequent coagulation of cellular debris with mucus on the lamellar surface which ultimately cause suffocation. This later theory of Cu toxicity has been supported and is currently maintained by most workers in the field.

The evidence presented in the literature and results of the present study indicate that at threshold or incipient lethal concentrations copper can enter the body in significant amounts. Furthermore, histological and behavioral observations indicate that fish exposed to these concentrations suffer from several symptoms of Wilson's Disease and damage to sensory systems. It

is therefore suggested that the primary mode of toxicity at the very sensitive and environmentally important concentrations is internal by impairing metabolic pathways, hemopoesis, sensory mechanisms, nervous transmission, neuromuscular coordination and muscular activity.

The Relationship of this Work to Water Quality Standards

Much of this work may give further insight into understanding copper and acid toxicity as they relate to establishing environmental quality controls. The copper ILC50 determined in the artificial freshwater used in the present study is substantially lower than the threshold values previously reported for salmonids in water with similar hardness and alkalinity (Lloyd and Herbert, 1962; EIFC, 1976, (168 hour LC50)). There are two possible explanations for these results: (1) The artificial freshwater is lacking inorganic anions besides carbonates which may exist in various test waters and are known to complex copper such as thiosulphate (Nishikawa and Tabata, 1969) and phosphates (Andrew et al., 1977) and subsequently reduce toxicity. (2) The turnover rate in the testing chambers used in this experiment was 2 to 3 times faster than the rates reported in the literature. This allows less time for mucus, feces or uneaten food to accumulate and chelate significant amounts of copper. The high turnover rate could be especially important at the beginning of the toxcity tests when copper was first being added, as the maximum concentration of copper was reached in approximately 4 hours. This gave the fish very little time to

physiologically adjust to copper. This explanation is supported by the observation that trout acclimate to copper in a remarkable short time and the mucus secreted in response to copper exposure has a great capacity to chelate copper.

The ILC50 in tap water was not as high as predicted by Lloyd and Herbert (1962), perhaps due to the rapid flow rate. However, it is significantly higher that the ILC50 obtained in the artificial freshwater, probably due to the higher concentration of phosphates and the addition of thiosulphate as a dechlorinator. The demonstration that trout have a remarkable ability to acclimate to copper also has important management implications.

The history of exposure of the test individuals is an important variable in assessing the toxicity of copper and possibly other heavy metals. As indicated in Figure 3 previous exposure to even minute concentrations of copper increased the fishes tolerance and preliminary exposure to .5 toxic units nearly doubled the resistance of fish to copper.

These results further suggest that differences in turnover rates of testing chambers may be of significant consequence to the results even when these rates exceed the minimum values recommended by A.P.H.A. (1975) and Sprague (1969).

Organic compounds have been found to affect copper toxicity. Stiff (1971b) found that single amino acids formed the major complex with copper except in very alkaline waters where carbonate (CO_3^{--}) is the most important chelator. A single amino acid, glycine, and undefined humic substances in concen-

trations of 2 to 5 mg/l were found to reduce copper toxicity to rainbow trout (Shaw and Brown, 1974).

The ILC50 for acid pH is similar to values reported in the literature (Lloyd and Jordan, 1964; Menendez, 1976). In the present studies toxicity tests were conducted for 15 days.

It is apparent from the results of my experiments and those reported by Lloyd and Jordan (1964) that at least 15 to 18 days is required to establish an ILC50 for Cu and low pH. In the present study mortality in the copper toxicity tests continued until the 9th day, mortality in the acid tests began on the 4th day continued until the twelfth day and the mortality in the copper-acid experiment was greatest between the eighth and twelfth day and continued until the fifteenth day.

These data indicate that the mortality rate varies with the different pollutants and combinations thereof. Therefore, the standard 96 hour LC50 may be dangerous underestimation of the toxicity of these pollutants.

The presence of other heavy metals, such as zinc and cadmium, even in low concentrations may also cause an underestimation of copper toxicity through additional or synergistic interactions (EIFAC, 1976; Sprague, 1969).

With the ultimate goal of establishing safe concentrations of pollutants, laboratory bioassays and timed LC50 tests may not be valuable in setting safe concentrations in various polluted waters.

Thus, there is accumulating evidence that cupric ion is the major toxic species of copper and calcium hardness, inorganic

alkaline compounds, organic substances, previous exposure of fish to copper, the flow or exposure rate of copper and ambient pH are all significant variables affecting copper toxicity.

Based on this information it is suggested that the determination of critical levels of copper and other heavy metals can only be done accurately by conducting bioassays to establish incipient LC50 values using the particular waters in question and fish which have been exposed to background levels of copper proportional to that found in such waters.

Potential for Further Research

This study has identified several areas which warrant further research. The modes of toxicity for copper appear to be different at different concentrations. At incipient lethal levels the absorption of copper into the fish and subsequent deposition in tissues with a high metabolic rate may be the primary mode of toxicity. The rates of absorption, the possible impairment of cellular and tissue functions by excessive amounts of copper will give further insight into the possible internal mode of toxicity. Such an assay could be done to test the action of copper on presynaptic membranes. With the apparent ability of copper to mimic calcium copper may act as mercury does in reacting with the sulfhydryl groups of the membrane proteins to cause the unintentional release of ACh. It is also postulated that at incipient lethal levels severe histological damage to the gills does not occur as it does with the 96 hour or shorter LC50 values (McKim and Benoit, 1971). This is sup-

ported by preliminary observations that respiratory rates gener-
ally return to preexposure values even prior to death at inci-
pient lethal levels.

The increased ability of trout to excrete excessive amounts
of copper which has entered the blood is suggested to be a
primary mechanism of acclimation. Determining copper and ceru-
loplasmin levels in the blood and urine may identify such a
phenomenon.

The specificity, structure, and mechanism by which copper,
and possibly other heavy metals bind to the mucoprotein is
another subject of potential research.

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APPENDIX I.   A TEST TO DETERMINE THE INFLUENCE OF MUCOPROTEIN

BINDING TO THE MEMBRANE OF THE CUPRIC ION ELECTRODE.

The addition of solutions of mucus to various copper standards immediately reduced the cupric ion activity of the copper standard. To insure that this was not an indirect effect due to the mucoprotein binding to the membrane and thus reducing the passage of cupric ions the electrode was polished, rinsed in .01 M sulphuric acid and then used to make a standard curve for copper. The electrode was then placed in a solution with a known concentration of copper and mucus. After stabilization the mV reading was noted and the electrode was held in this solution for approximately 12 hours. At this time a second and nearly identical reading was taken. The electrode was then rinsed with distilled water and placed in the known standard. This mV reading was almost identical to the previous reading. Thus, it was concluded that the results of titrations with mucus was due to the effect of copper binding to the mucus in solution and suspension rather than the mucus binding to the membrane.

APPENDIX II. Mortality curves obtained from the experiment

designed to test the lethal interaction of

copper and acid pH.

Figure 1.   The toxic interaction of acid pH and copper. Two testing

chambers were maintained at a specific pH while the

copper concentration was varied for each. In this way

4 combinations of acid pH and a high and low copper

concentration were tested simultaneously. Two trials

were required to establish an ILC_{50} at each pH value.

Three points are shown on each of the curves because

2 of the copper concentrations yeilded either 0 %

mortality or 100 % mortality and therefore only 1 of

those concentrations was used to establish the curve.
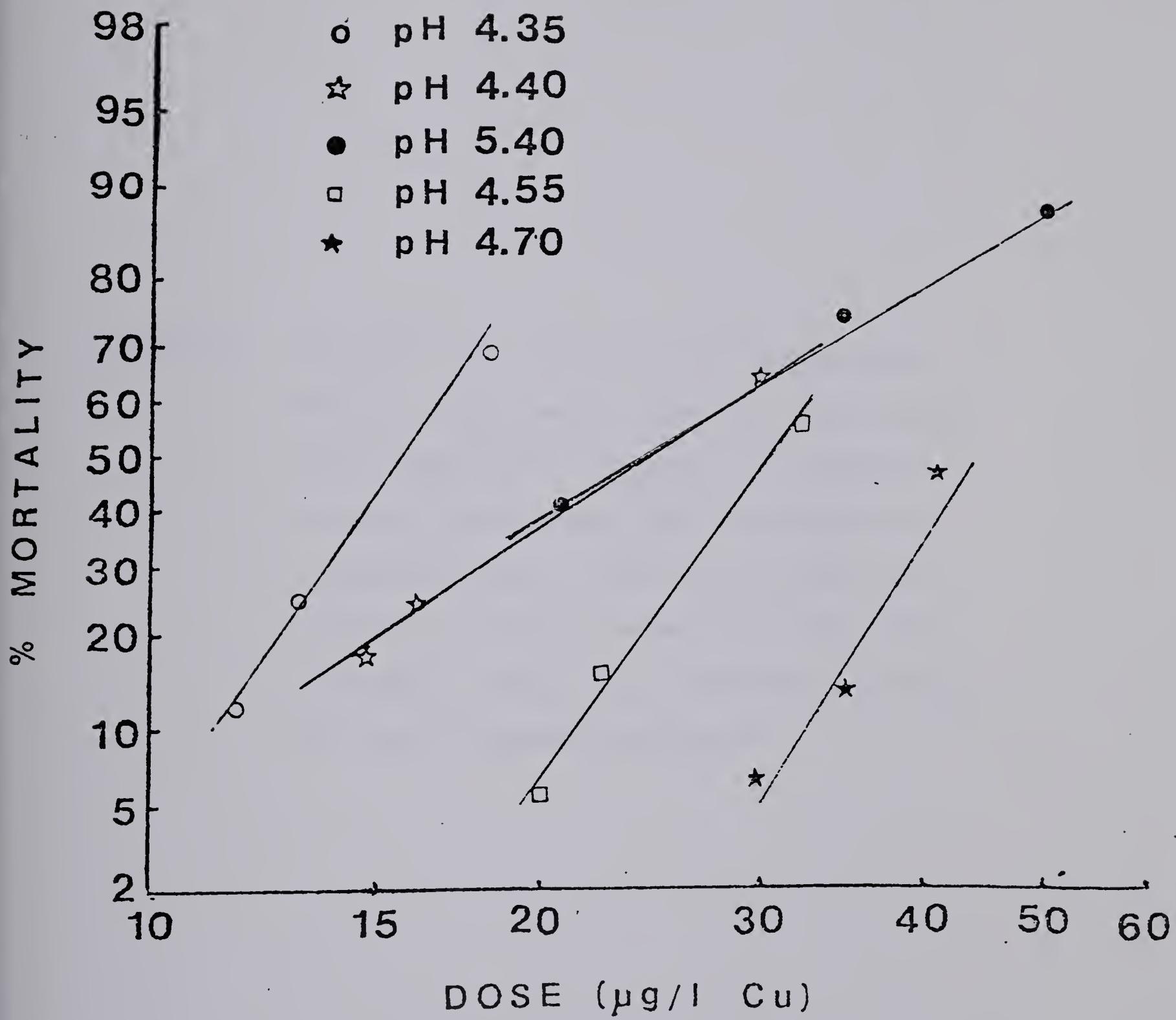
APPENDIX III. Mortality curves obtained for the hardness and
alkalinity experiments. These curves are plotted
on log-probit paper according to the method of
Litchfield and Wilcoxon (1949). The baseline
ILC50 established at a hardness of 50 mg/L and
alkalinity of 28 mg/L, served as the value for
intermediate alkalinity and intermediate hardness.
This curve is expressed on page 30.

Figure 1. The dose response curve for copper obtained in water
having a hardness of 12 mg/l and alkalinity of 10
mg/l (low alkalinity, low hardness). Error bars
indicate the 95 % confidence limits.
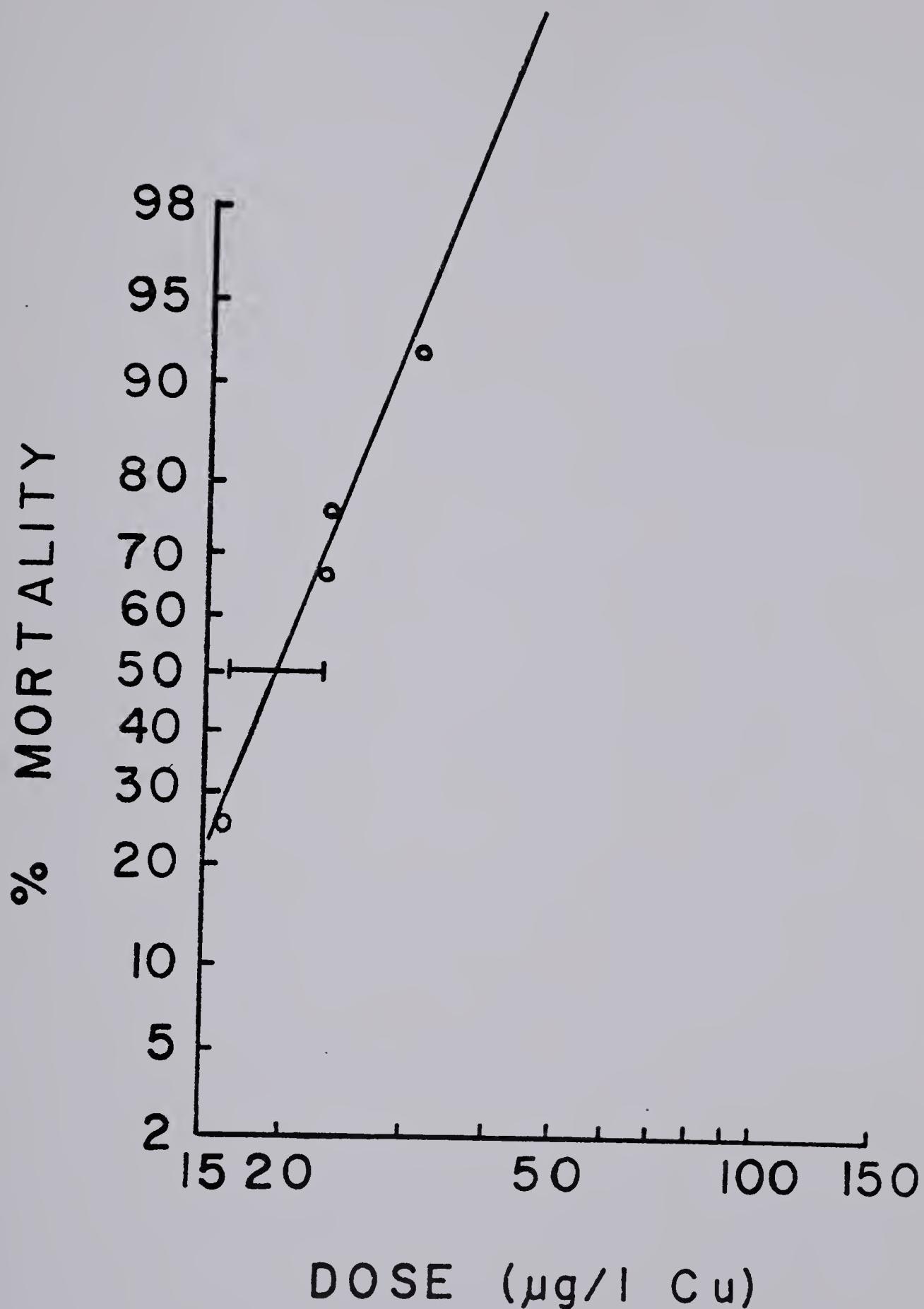
Figure 2. The dose response curve for copper obtained in water having a hardness of 100 mg/l and alkalinity of 10 mg/l (low alkalinity, high hardness). Error bars indicate the 95 % confidence limits.
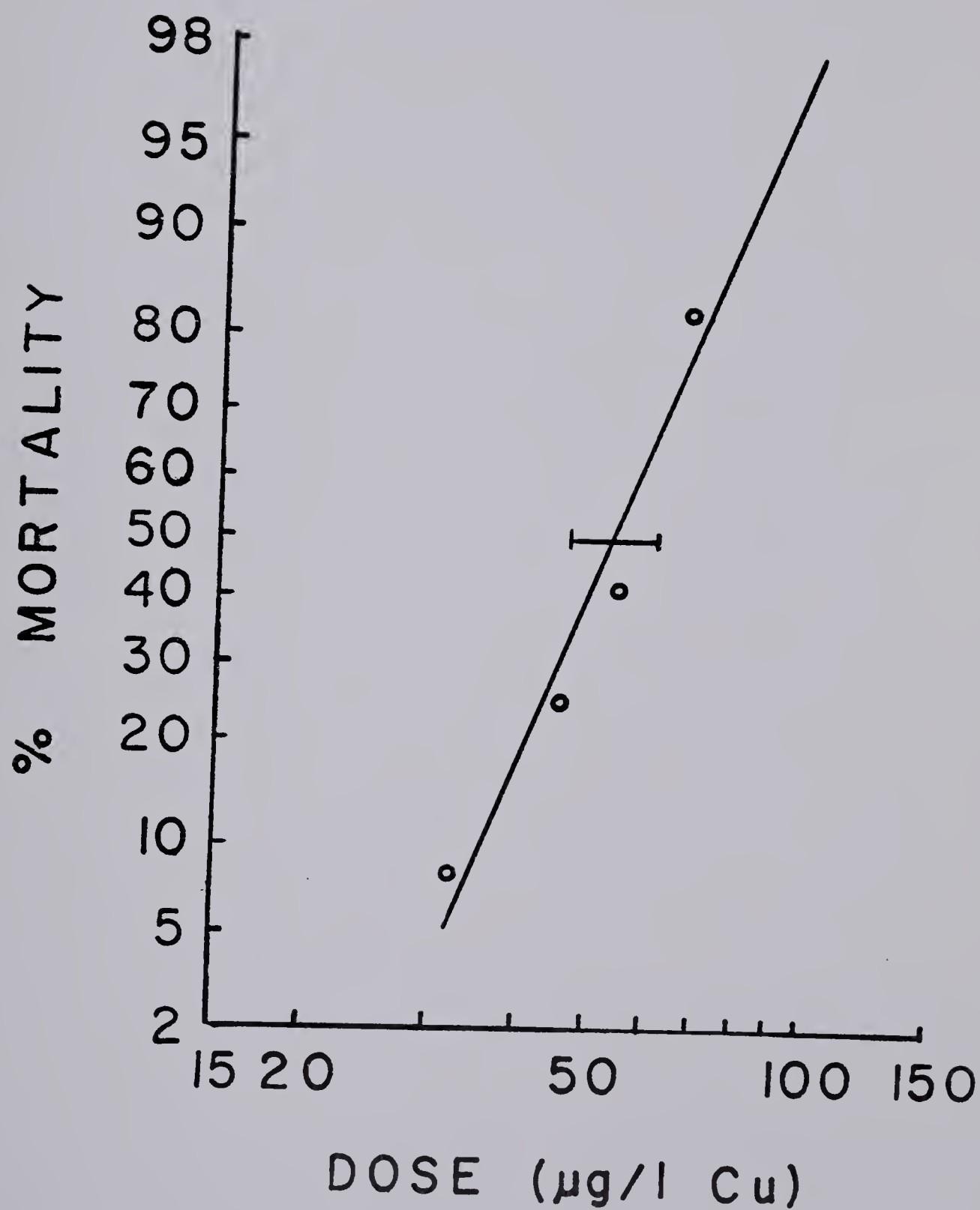
Figure 3. The dose response curve for copper obtained in water

having a hardness of 100 mg/l and alkalinity of 28

mg/l (Intermediate alkalinity, high hardness). Error

bars indicate the 95 % confidence limits.
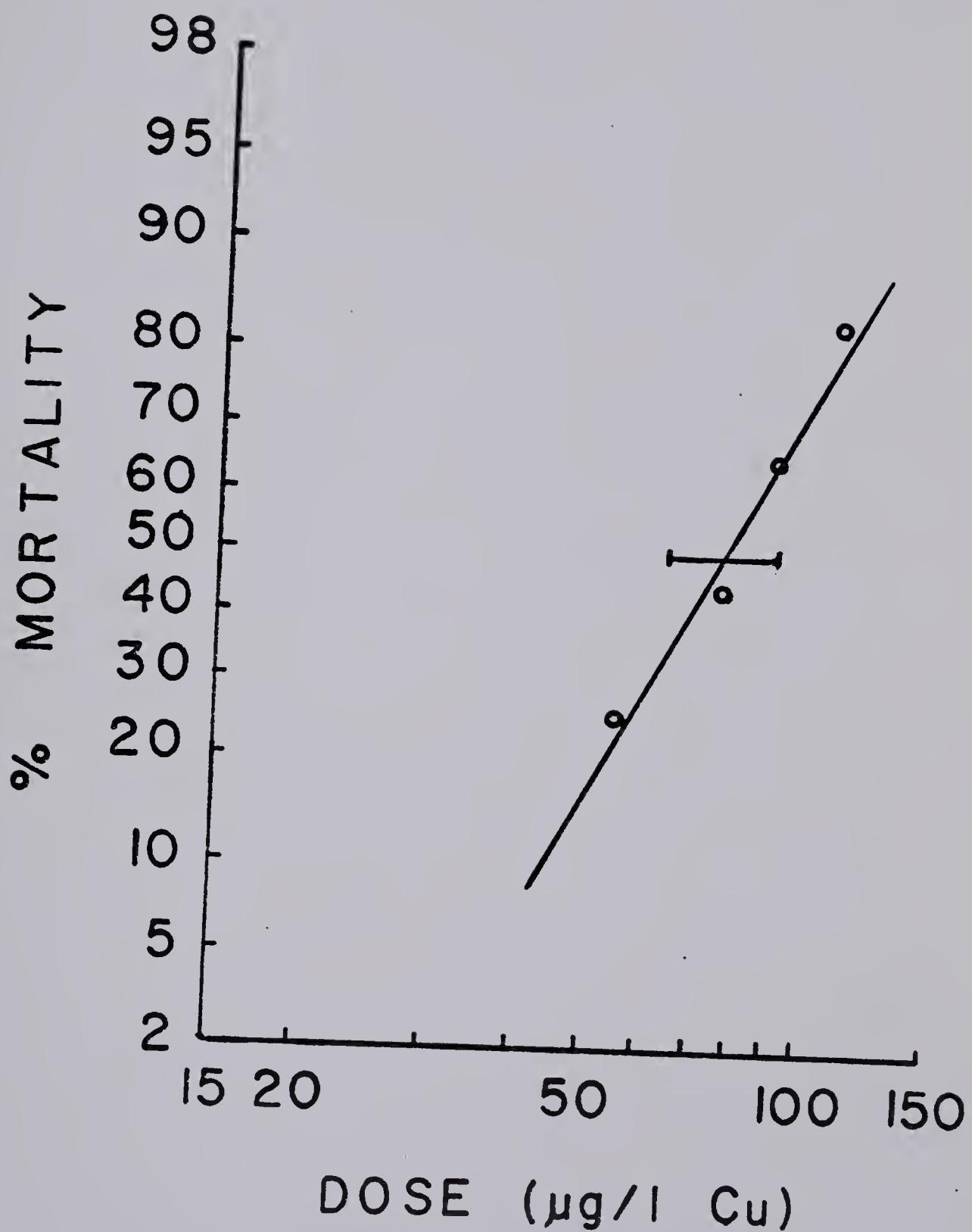
Figure 4. The dose response curve for copper obtained in water
having a hardness of 12 mg/l and alkalinity of 50
mg/l (high alkalinity, low hardness). Error bars
indicate the 95 % confidence limits.

DOSE (µg/l Cu)
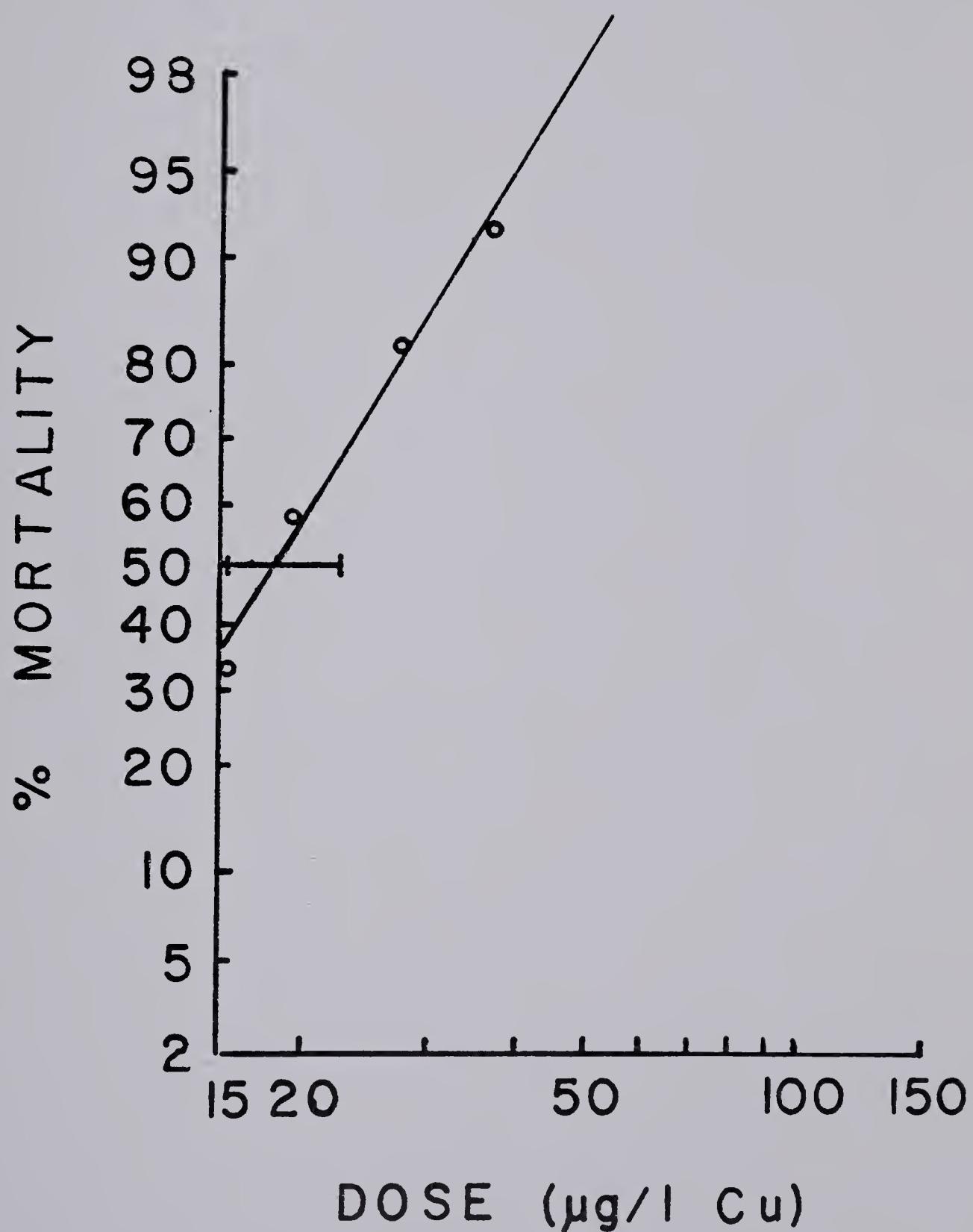
Figure 5. The dose response curve for copper obtained in water
having a hardness of 100 mg/l and alkalinity of 50
mg/l (high alkalinity, high hardness). Error bars
indicate the 95 % confidence limits.
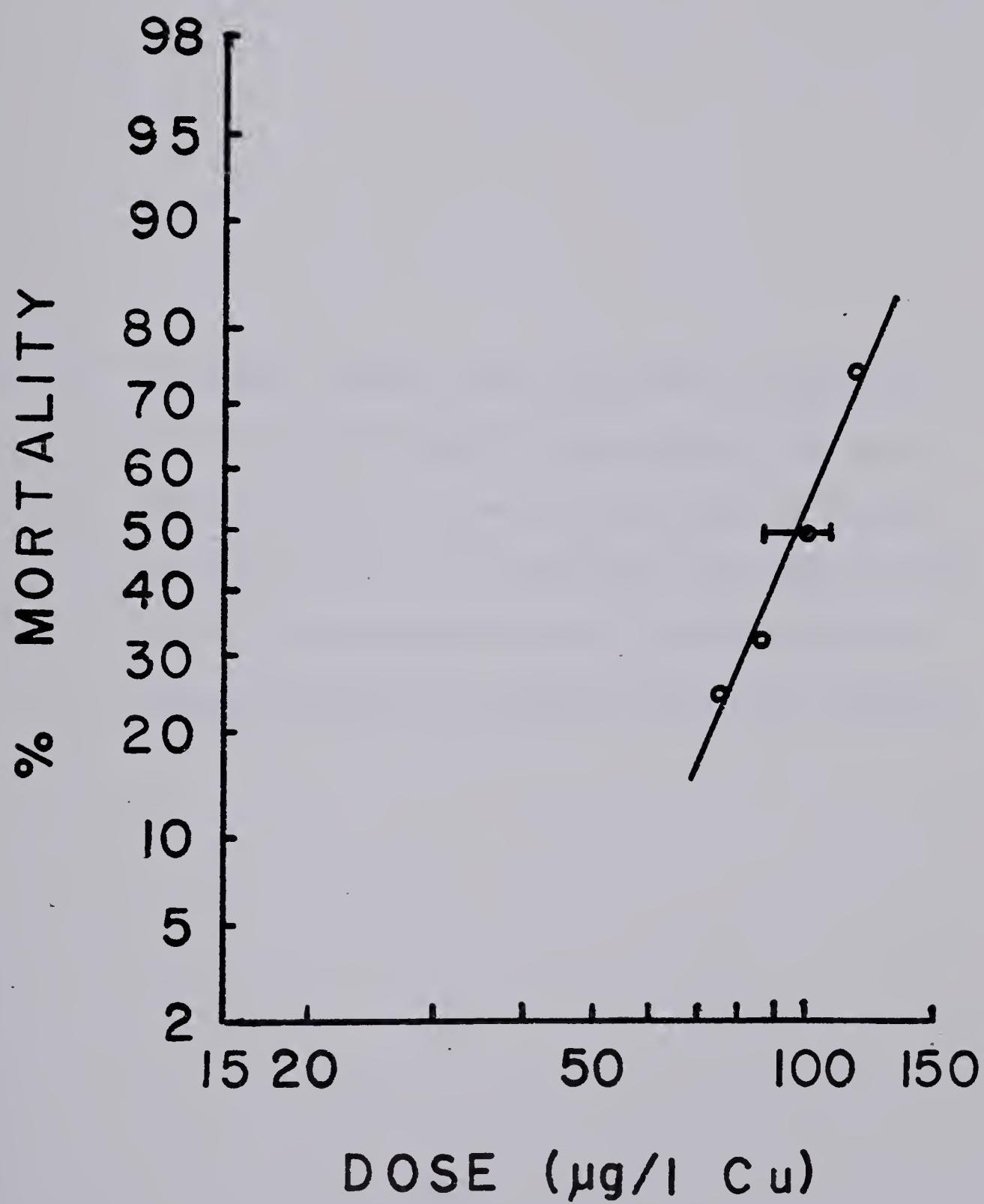
APPENDIX IV. Additional toxicity tests conducted to compare (1)

the toxicity of copper in dechlorinated tap water

which contained hardness and alkalinity in similar

concentrations as the artificial fresh water and (2)

the relative susceptibility of fingerling rainbow

trout to copper after aging and growth had occurred.

Figure 1. Mortality curve for copper using rainbow trout held

in dechlorinated tap water. The water had an average

hardness of 58 mg/L and an average alkalinity of 33 mg/L.

This water also contained sodium thiosulphate (1.2 mg/L)

which is added as a dechlorinater and which is a known

chelator of copper.
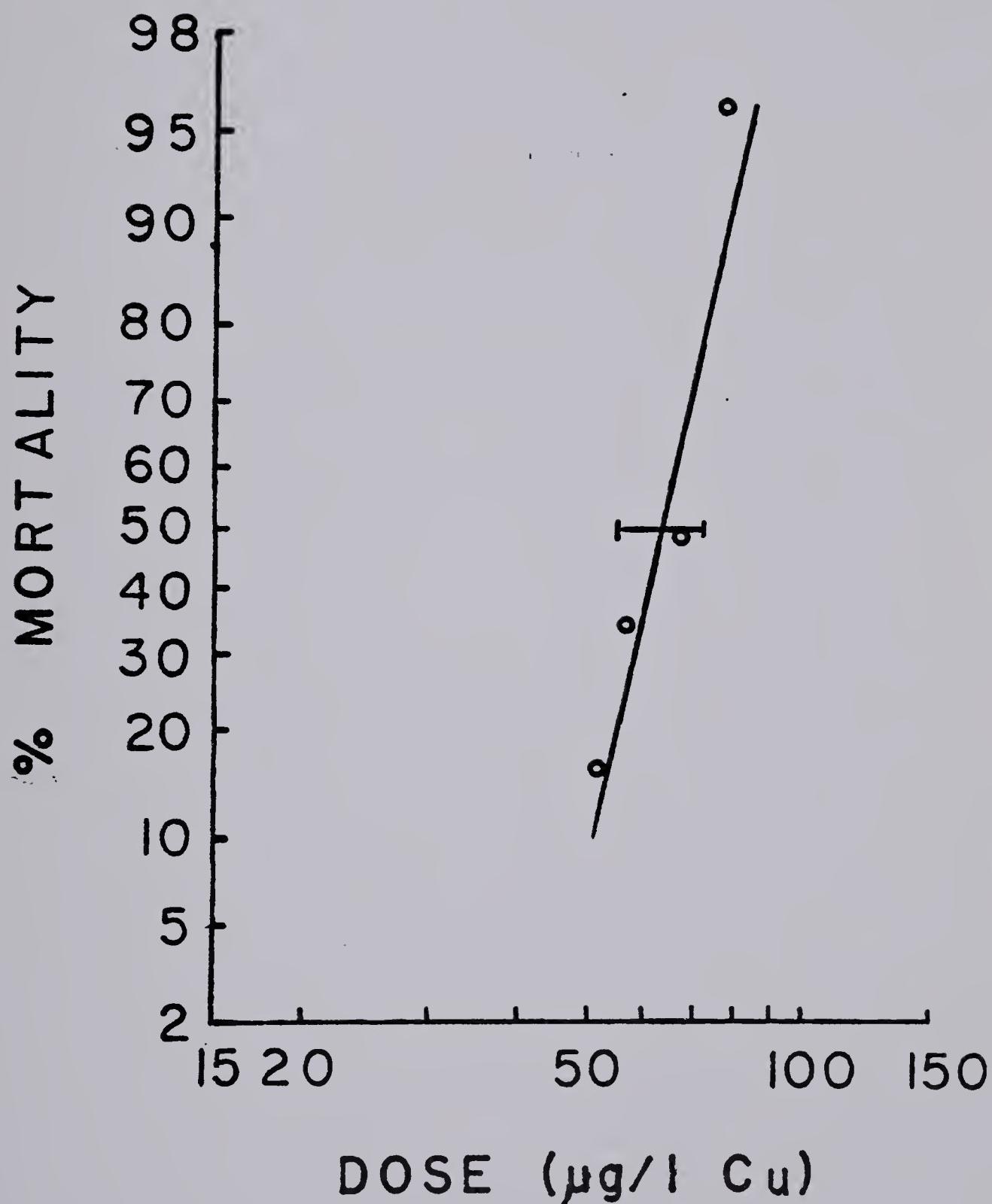
Figure 2. Mortality curve expressing the repeat ILC50 which was
conducted at the end of all other toxicity tests.
Artificial fresh water identical to that used in the
baseline ILC50 was used and fingerling trout from the
lot of fish obtained from the Sam Livingston Fish
Hatchery were used. These fish were 9 months older
and averaged 14 gms heavier (ranging from 17 to 29 gms)
than fish used to conduct the baseline ILC50. The
ILC50 was nearly identical to the baseline ILC50.
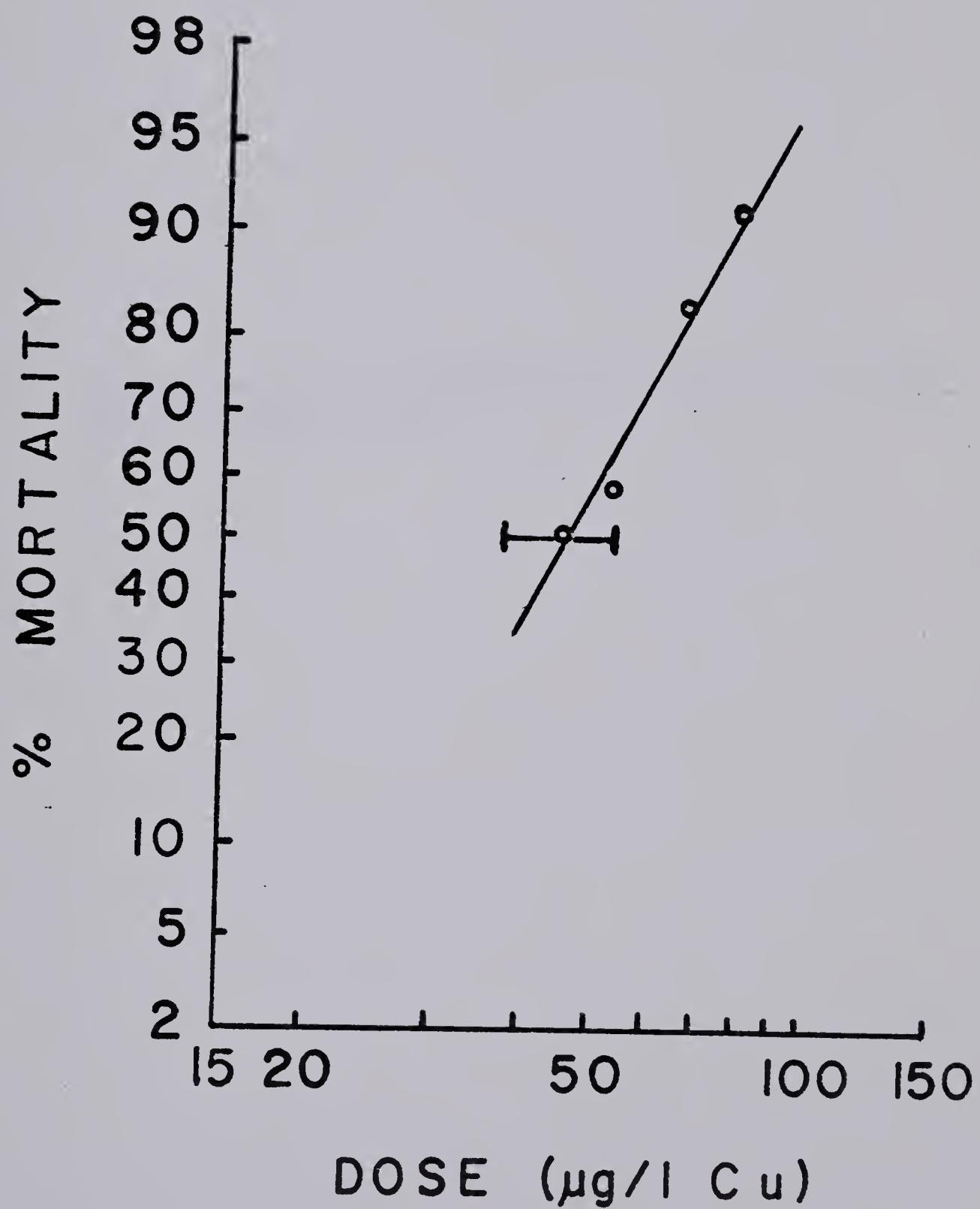
APPENDIX V.   An Hypothesis for the Mechanism Involved in an Internal
              Mode of Copper Toxicity

According to current theory, the arrival of an action potential at the presynaptic terminal causes a conformational change in the membrane which allows calcium ions to enter and facilitate the binding of synaptic vessicles to the outer membrane. If free copper ions are present, then their similarity to calcium in binding quality may allow them to mimic and thus add to the action of calcium causing greater ACh release, depolarization and subsequent contraction. Furthermore, if copper leaks or is sequestered into the cell it may be treated as calcium and stored in the sarcoplasmic reticulum with calcium. When a depolarization occurs, the Ca^{++} and possibly Cu^{++} enter the cytoplasm and bind to the troponin complex of the actin filament in the contractile apparatus. The efficient binding of calcium leads to a prolonged and steady contraction (Figure 9). However, if copper is present in the system, it may also bind to the troponin but apparently not as effectively or for as long a period of time. The result appears to be a rapid release and binding of copper with the actin filament and subsequent rapid contraction and relaxation of the contractile apparatus as evidenced by the copper exposed fish and the tracings in Figure 12.